

Assessment of sperm DNA integrity in workers exposed to styrene

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BACKGROUND: Occupational exposure to toxic agents may cause infertility, congenital anomalies or death in offspring, but few studies have evaluated DNA integrity in germ cells of male workers. We investigated sperm DNA integrity in individuals occupationally exposed to styrene. **METHODS AND RESULTS:** Semen samples were obtained from 46 male workers exposed to styrene and 27 unexposed controls (age range 18–45 years). Exposed individuals had worked for at least 2 years in the last 5 years and continuously for 6 months in factories producing reinforced plastics. The Comet assay was performed to evaluate DNA integrity in sperm, as well as semen quality analysis to assess sperm concentration and morphology. There were no differences in the results of the standard semen analysis between exposed subjects and the reference group. However, we found a significant difference ($P < 0.001$) in sperm DNA damage by the Comet assay between exposed subjects and the reference group. **CONCLUSIONS:** The Comet assay proved to be sensitive in detecting an alteration in DNA integrity in germ cells of workers exposed to styrene. This finding contributes towards the understanding of the importance of male occupational exposure within the context of genetic risk assessment in humans.

Key words: Comet assay/DNA damage/occupational exposure/semen analysis/styrene

Introduction

Male reproductive tract disorders have become an important public health issue, as they may cause miscarriages and abnormal outcomes in the offspring. In 20% of cases of couple infertility the problem is predominantly male and in up to 40% of men with sperm abnormalities, no specific aetiological factor is found (De Kretser, 1997). These disorders may be the consequence of environmental or occupational exposure to chemicals, radiation, toxicants and heat (Friedler, 1996). Semen quality analysis, the standard clinical approach to assess male reproductive capacity, can be considered a sensitive biological marker of exposure to toxicants at the work place (Wyrobek, 1993; Bigelow *et al.*, 1998). This analysis has shown that sperm concentration and total sperm count decline in workers exposed to different classes of toxic agents, such as dibromochloropropane and lead, and that sperm motility diminishes following exposure to high temperatures in subjects employed in the ceramics industry (Whorton *et al.*, 1977; Alexander *et al.*, 1996; Figa-Talamanca *et al.*, 1992). In addition, alterations in sperm morphology have been observed in trichloroethylene-exposed workers (Chia *et al.*, 1996), and occupational exposure to petrochemical compounds may aggravate the adverse effect of smoking on semen quality

(Wang *et al.*, 2001). Furthermore, environmental factors, particularly exposure to pesticides and solvents, have been associated with considerable changes in sperm parameters in an infertility consulting population, suggesting that testicles are one of the most vulnerable organs to environmental physical and chemical agents (Oliva *et al.*, 2001).

Environmental or occupational exposure can also lead to abnormal reproductive outcomes by altering the integrity of genetic material, at chromosome or DNA level, in male germ cells (Wyrobek, 1993). Even though a large amount of data is not yet available, smoking, alcohol and caffeine consumption, and anticancer drugs have been reported to cause aneuploidy in human sperm (Robbins *et al.*, 1997; Martin *et al.*, 1999), while a lower sex ratio at birth has been associated with increasing serum 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentrations in Italian men after the Seveso accident (Mocarelli *et al.*, 2000).

Recently, a modified version of the single cell gel electrophoresis (SCGE)—also known as the Comet assay—has been devised that can assess sperm DNA integrity (McKelvey-Martin *et al.*, 1997). This assay is a rapid, simple and sensitive technique for measuring DNA damage of individual cells, which at present is usefully employed in human biomonitoring

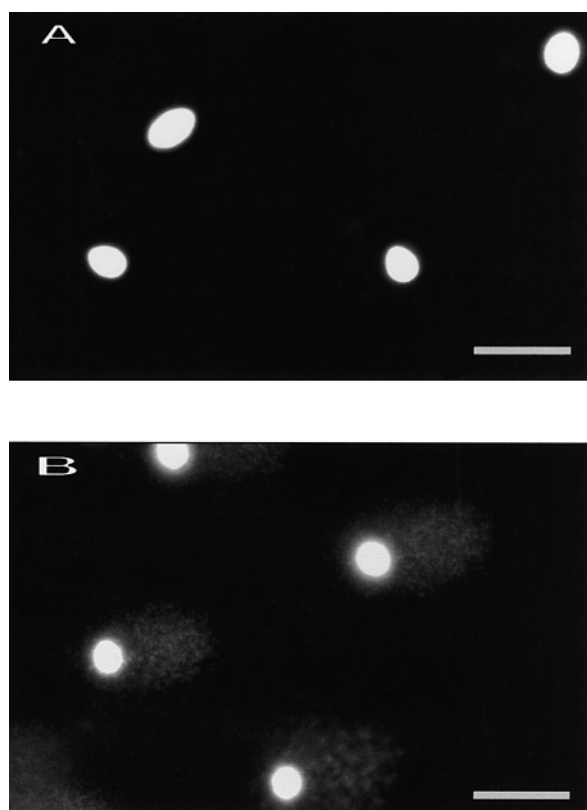


Figure 1. Sperm cells processed using the Comet assay, stained with ethidium bromide, showing: (A) cells with intact, undamaged DNA and (B) cells with a high degree of DNA damage exhibiting the typical 'comet tail' appearance. Scale bar = 20 μm .

studies conducted only on somatic cells (Kassie *et al.*, 2000). In the presence of strand breaks, the method allows the relaxed loops of DNA to migrate and appear as a 'comet tail' when cells are subjected to alkaline electrophoresis and then stained with a fluorescent DNA-binding dye (Figure 1). Intensity and length of the comet tail, depending on the frequency of DNA fragments, or the relative percentage of undamaged DNA in the intact nucleus (comet head) can be easily measured by a computer-based image analysis (Fairbairn *et al.*, 1995).

Styrene is one of the most important monomers worldwide and is extensively used in the manufacture of reinforced plastics, protective coatings, synthetic rubbers and polyesters (International Agency for Research on Cancer, 1994). In highly exposed subjects, styrene induces skin or respiratory tract irritation and central nervous system depression (Bond, 1989). In addition, this chemical seems to affect the correct development and maturation of male germ cells by interacting with the neuroendocrine hypothalamic–pituitary–gonadal axis (Takao *et al.*, 2000). Although there is inadequate evidence of the carcinogenicity of styrene in humans, the IARC classified styrene into Group 2B and styrene 7,8-oxide, its main intermediary metabolite, into Group 2A (probable human carcinogen) (International Agency for Research on Cancer, 1994). A large number of genotoxicity studies have been performed in somatic cells of workers exposed to styrene and increases in the frequency of cells with chromosome anomalies or with DNA damage have been reported (Tates *et al.*, 1994; Vodicka *et al.*, 1999, 2002). The only two reports available in

the literature on the effects of styrene on male reproductive function of occupationally exposed subjects evaluated exclusively conventional semen quality parameters (Jelnes, 1988; Kolstad *et al.*, 1999).

In the present study we assessed DNA fragmentation in the sperm of a group of male workers occupationally exposed to styrene in three different areas of Tuscany (Italy) and of a reference unexposed group, using the Comet assay in addition to the standard sperm quality analysis (sperm concentration and morphology). We also measured urinary concentration of mandelic acid (MA), the main urinary metabolite of styrene, as a biomarker of recent exposure to this chemical.

Materials and methods

Subjects

The survey was conducted in ten Italian factories located in three different districts (Lucca, Pisa and Livorno) of the region of Tuscany (Italy), identified as Area A, Area B, and Area C. Each of these districts was under the control of a local Medical Department, responsible for recruitment of subjects, collection of biological samples and providing demographic and exposure data. We stratified styrene-exposed individuals according to the districts in order to avoid the introduction of confounding factors (such as differences in lifestyle, working conditions and differences in socioeconomic background). In these factories, ~70 male subjects are employed in the production of fibre-reinforced plastics (boat building factories). Workers are mainly involved in hand-spraying lamination processes using unsaturated polyester. Eligible subjects had to have been employed for ≥ 2 years in the last 5 years and continuously for 6 months before biological sampling. No comparison between intermittent and continuous exposure was possible since only those subjects meeting both criteria were enrolled. To our knowledge, lamination workers did not regularly wear protective equipment. The reference group included healthy male volunteers of comparable age, with no previous history of styrene exposure, living in the same areas. All subjects were interviewed to complete a detailed questionnaire on their personal, occupational and medical history and lifestyle. Preliminary information on recent exposure to X-rays, viral infections or genitourinary tract inflammatory disorders experienced in the last 3 months or current use of medicinal products were used as criteria to exclude subjects from the study. Semen samples were obtained from a total of 46 styrene-exposed workers and 27 unexposed subjects.

Informed consent for participation in the study was obtained from each subject and the study was approved by the local Ethics Committees.

Urinary MA determination

Urine specimens were obtained from styrene-exposed workers once at the end of the work shift, in the same week as the semen sample collection, and analysed for MA levels using a standard method described elsewhere (Poggi *et al.*, 1982). MA values were expressed in mg/g of creatinine in order to compare different concentrations of MA after correction of the results for dilution of the urine, urinary flow and body mass (World Health Organization, 1996; Symanski *et al.*, 2001).

Semen analysis

Semen samples, obtained by masturbation after 3 days of recommended sexual abstinence, were allowed to liquefy at 37°C, following

which an aliquot was removed in order to construct a conventional semen profile composed of ejaculate volume (in ml), sperm concentration ($\times 10^6/\text{ml}$) and normal morphology (%) according to published guidelines (World Health Organization, 1992).

Determination of DNA integrity using a modified alkaline single cell gel electrophoresis (Comet) assay

The modified alkaline Comet assay for sperm (McKelvey-Martin et al., 1997) was carried out according to the following procedure. Fully frosted microscope slides were covered with 1% normal melting point agarose (Sigma-Aldrich, Italy). About 10 μl of human sperm in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS; Sigma-Aldrich) were mixed with 85 μl of 0.5% low melting point agarose (Agarose wide range; Sigma-Aldrich) at 37°C, under yellow light to prevent further induced damage to DNA. This cell suspension was rapidly pipetted on top of the first agarose layer, covered with a coverslip and allowed to solidify at 4°C for 5 min. A final layer of 0.5% low melting point agarose was added to the slide and allowed to solidify at 4°C for 10 min.

The cells were then lysed by immersing the slides in a coplin jar containing freshly prepared cold lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA , Tris 10 mmol/l, 10% DMSO with 1% Triton X-100 (pH 10; Sigma-Aldrich) for at least 1 h at 4°C. Then slides were incubated overnight at 37°C with 100 $\mu\text{g}/\text{ml}$ proteinase K (Sigma-Aldrich) in order to remove protamines that otherwise impede DNA migration through the agarose.

A horizontal gel electrophoresis tank was filled with alkaline electrophoresis solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH 12.5) at room temperature. The slides were placed into this tank side by side with the agarose end facing the anode and with the electrophoresis buffer at a level of ~0.25 cm above the slide surface. The slides were left in this high pH buffer for 20 min to allow DNA to unwind. The DNA fragments were then separated by electrophoresis for 10 min at 25 V adjusted to 300 mA. After electrophoresis the slides were flooded with two changes of neutralization buffer (0.4 mol/l Tris, pH 7.5) for 5 min each. This removed any remaining alkali and detergents, which could have interfered with staining. The slides were drained before being stained with 100 μl of 20 $\mu\text{g}/\text{ml}$ ethidium bromide (Sigma-Aldrich). Coded slides were viewed using an Eclipse E800 Nikon epifluorescence microscope equipped with a filter for ethidium bromide visualization. For each sample, 100 randomly selected sperm nuclei were evaluated by an image analysis system using Komet 4.0 software (Kinetic Imaging, Liverpool, UK). The relative tail fluorescence (tail DNA percentage, TP) and the olive tail moment (OTM, the product of tail length and percentage of DNA in the tail), expressed as mean from the 100 cells scored per donor, were used as a measure of primary DNA damage.

Statistical analysis

Data were analysed using the Statgraphics Plus software package for Windows (version 5.0). Differences between exposed subjects and unexposed controls were evaluated by multifactor analysis of variance after including smoking habits and age in the model as confounding factors. A multiple comparison procedure (Bonferroni's method) was also performed to detect differences among exposure sampling areas and the reference group. The relationship between DNA damage and sperm concentration or urinary MA levels was tested by first order regression analysis.

Results

Demographic characteristics of the study population

Table I shows the composition and demographic characteristics (age and smoking habits) of the study population stratified

according to exposure and recruitment area. Unexposed and exposed groups consisted of 27 and 46 subjects respectively; their mean (\pm SD) ages were similar (32.0 ± 7.4 versus 31.5 ± 5.3 years). The percentage of smokers was different, being 63.0% in unexposed controls and 48.2% in laminators. Subject mean age and smokers/non-smokers distribution in the three exposure areas deviated from those of the group *in toto*. However, appropriate adjustments for unbalanced size and differences in the proportion of subjects when matching exposed subjects and controls for confounding factors were introduced by multivariate analysis.

Exposure indicators

Table II shows the extent of styrene exposure expressed as years of employment and urinary MA concentration. Workers from Area A and Area C were employed, on average, for 6.8 and 7.0 years respectively, whereas laminators of Area B had an average styrene exposure of 12.4 years. Workers from Area A had MA values ranging from 15.7 to 1428.7 mg/g creatinine with a median value of 313.0 mg/g creatinine. The levels of styrene exposure were lower in the laminators from Area C (5.8–341.0 mg/g creatinine, median of 52.9), whereas the median value was 199.1 (range 24.4–1202.7 mg/g creatinine) in subjects from Area B was intermediate between the two other areas. In addition, 28.3% (13/46) of all the exposed individuals had urinary MA values >420 mg/g creatinine. This value at the end of the work shift represents the biological exposure value corresponding to 85 mg/m^3 (20 ppm) of styrene at the working place (TLV-TWA) American Conference of Governmental Industrial Hygienists, 2000), calculated in accordance with Lauwerys and Hoet (Lauwerys and Hoet, 1993).

Semen analysis

Table III reports the results of semen analysis. In the exposed and control groups, semen sample volume was in the range of normality (2–6 ml) in 41/46 (89.1%) and 22/27 (81.5%) subjects respectively. Sperm concentration values did not differ between exposed and unexposed subjects: only Area B subjects presented a significantly lower concentration. In Area A and Area B we detected 2/17 and 4/20 oligozoospermic samples; two laminator samples, one for each area, were found to be azoospermic, whereas all samples from Area C had normal sperm concentration. In the control group, 4/27 samples had sperm concentration values $<20 \times 10^6/\text{ml}$. Only two subjects of the study population, one in the control group and one in the exposed group, exhibited a high percentage ($>70\%$) of sperm with morphological alterations.

Comet assay

The results related to sperm DNA integrity, as measured by the Comet assay, are reported in Table IV. Compared with the control group, after adjusting for confounding factors, workers exposed to styrene showed significantly elevated DNA migration ($P < 0.001$): TP 10.9 ± 3.0 versus 7.4 ± 2.3 , and OTM 1.5 ± 0.6 versus 0.8 ± 0.4 . As expected, TP and OTM values were strongly related to each other. When the data were viewed according to exposure areas, for each area OTM mean values

Table I. Demographic characteristics of the study population

	Area A (<i>n</i> = 17)	Area B (<i>n</i> = 20)	Area C (<i>n</i> = 9)	All exposed (<i>n</i> = 46) ^a	Unexposed (<i>n</i> = 27)
Mean (SD) age (years)	28.8 (4.8)	34.2 (5.2)	30.4 (5.2)	31.5 (5.3)	32.0 (7.4)
Smoking habit					
Smokers (%)	14 (82.4)	13 (65.0)	2 (22.2) ^b	29 (63.0)	13 (48.2)
Non-smokers (%)	3 (17.6)	7 (35.0)	7 (77.8) ^b	17 (37.0)	14 (51.8)

^aArea A + Area B + Area C.^b*P* < 0.05; Bonferroni's multiple comparison procedure: Area C versus Area A, Area B and Unexposed.**Table II.** Exposure data of styrene workers

	Area A (<i>n</i> = 17)	Area B (<i>n</i> = 20)	Area C (<i>n</i> = 9)	All exposed (<i>n</i> = 46) ^a
Mean (SD) period of employment (years) ^b	7.0 (4.2)	12.4 (6.5) ^c	6.8 (2.9)	9.2 (5.7)
Median (range) values of urinary mandelic acid (mg/g creatinine) ^d	313.0 (15.7–1428.7)	199.1 (24.4–1202.7)	52.9 (5.8–341.0)	173.6 (5.8–1428.7)

^aArea A + Area B + Area C.^bIn reinforced plastic factories.^c*P* < 0.05; Bonferroni's multiple comparison procedure: Area B versus Area A and Area C.^dUrinary mandelic acid concentration of 420 mg/g creatinine corresponds to an estimate of 85 mg/m³ of styrene at the workplace (20 ppm) (TLV-TWA, established by the American Conference of Governmental Industrial Hygienists, 2000), calculated in accordance with Lauwerys and Hoet (1993).**Table III.** Results of the semen quality analysis

	No. (%) of subjects				
	Area A (<i>n</i> = 17)	Area B (<i>n</i> = 20)	Area C (<i>n</i> = 9)	All exposed (<i>n</i> = 46)	Unexposed (<i>n</i> = 27)
Semen volume					
<2 (ml)	3 (17.7)	2 (10.0)	0 (0.0)	5 (10.9)	5 (18.5)
≥2 (ml)	14 (82.3)	18 (90.0)	9 (100.0)	41 (89.1)	22 (81.5)
Sperm concentration					
<20 (×10 ⁶ /ml)	3 (17.7)	5 (25.0)	0 (0.0) ^b	8 (17.4)	4 (14.8)
≥20 (×10 ⁶ /ml)	14 (82.3)	15 (75.0)	9 (100.0) ^b	38 (82.6)	23 (85.2)
Sperm morphology					
% of normal forms <30	1 (5.9)	0 (0.0)	0 (0.0)	1 (2.2)	1 (3.7)
% of normal forms ≥30	16 (94.1)	20 (100.0)	9 (100.0)	45 (97.8)	26 (96.3)

^aArea A + Area B + Area C.^b*P* < 0.05; Bonferroni's multiple comparison procedure: Area B versus Area A, Area C and Unexposed. Age and smoking habit controlled.**Table IV.** Results of the Comet assay

	All exposed (<i>n</i> = 44) ^a	Unexposed (<i>n</i> = 27)	<i>P</i> ^b
Mean (SD) tail DNA percentage ^c	10.9 (3.0)	7.4 (2.3)	0.001
Mean (SD) olive tail moment ^c	1.5 (0.6)	0.8 (0.4)	0.0001

^aArea A + Area B + Area C.^bAge and smoking habit controlled.^cCalculated scoring 100 sperm nuclei per subject.

were significantly higher than that of the control group (Figure 2). In styrene-exposed workers, no significant correlation was found between the two DNA damage parameters considered

and years of employment in reinforced plastic factories or urinary MA concentration. Moreover, DNA damage of the study population increased significantly (*P* < 0.05) when

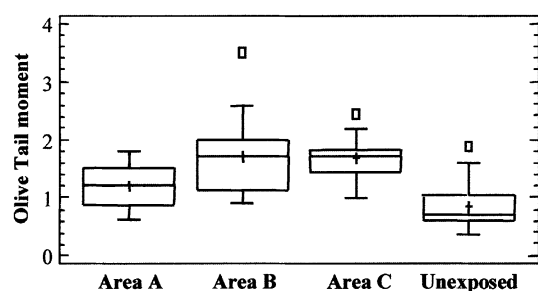


Figure 2. Box-and-whisker plots of the levels of sperm DNA integrity as measured by olive tail moment (OTM) in styrene-exposed workers from three geographical areas and unexposed controls. Multiple range test (Bonferroni's method) was performed to detect differences between sampling areas and the reference group. Comet assay results show that workers belonging to each area have significantly higher values of OTM than unexposed subjects ($P < 0.01$). The box groups the 50% data distributed around the median. The remaining 50% is represented by the whiskers (the vertical dash represents the average value, the square identifies outlier values).

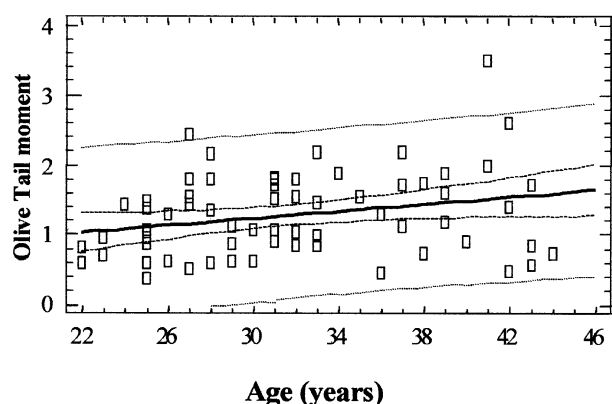


Figure 3. Sperm DNA integrity, as measured by olive tail moment, of the total study population (exposed and unexposed subjects) plotted against age. A significant correlation can be observed ($r^2 = 0.354$, $P < 0.05$).

chronological age increased (Figure 3), while no effect of smoking habit was observed. Subjects with reduced sperm concentration had a tendency to show high levels of DNA damage (data not shown).

Discussion

Styrene is a widely used chemical and thousands of workers worldwide are exposed to this compound every day, predominantly via inhalation, but also via contact with the skin and subsequent absorption. It is therefore imperative to assess the risks involved in human exposure to this substance. To our knowledge, this is the first study on styrene-exposed workers in which a classical approach for assessing exposure effect on male germ cells (sperm quality analysis) has been implemented with the Comet assay, a molecular cytogenetic technique designed specifically to measure DNA breakage in individual cells. In this study, biological samples were obtained from 46 styrene-exposed laminators and 27 healthy controls; the occupational exposure level to styrene was assessed by the amounts of urinary MA.

In our study, the proportion of exposed subjects with abnormal sperm concentrations (azoospermia or oligozoospermia) and the mean values of sperm count and sperm morphology were not different from those in healthy controls. An increased proportion of sperm with abnormal morphology was observed in Danish workers producing reinforced plastics compared with matched controls (Jelnes, 1988). The difference in sperm abnormality between the workers investigated by Jelnes and our hand-laminators may reflect either natural inter-individual variability or could be associated with different styrene exposure levels, since the styrene concentrations reported for the Danish workers were ≥ 5 -fold higher than those in our three cohorts (almost 100 ppm in the former versus < 20 ppm in the latter report). Moreover, a decline in sperm density, but not in other semen parameters, was detected after a 6 month styrene exposure in another study (Kolstad *et al.*, 1999). However, authors reported that changes were not related to urinary MA concentration or styrene air concentration at the work place.

We measured the extent of DNA damage in sperm in terms of migrated DNA fragments by using the TP and the OTM, two of the most commonly used parameters in the Comet assay. Interestingly, we detected increased levels of both TP and OTM in each of the three styrene-exposed worker groups as compared with the reference controls. OTM, incorporating a measure of both the smallest detectable size of migrating DNA and the number of broken pieces, appeared more sensitive than TP (Fairbairn *et al.*, 1995). Similar parameters for evaluating DNA damage have also been used in another study on sperm cells in IVF treatment study. The authors recommended the employment of at least two parameters reflecting DNA damage (although they are interrelated) (Morris *et al.*, 2002), since it cannot be ruled out that the amount of DNA released from the head and the length of DNA migration may be increased independently in some instances (Olive, 1999).

The evidence of styrene-induced DNA fragmentation is a novel finding in male germ cells of occupationally exposed individuals. The maintenance of DNA integrity is absolutely necessary for the accurate conveyance of genetic material. Although DNA damage does not necessarily affect sperm fertilization ability (Ahmadi and Ng, 1999), the biological significance of fertilization of an oocyte by a sperm carrying high amounts of DNA damage is not known. This may interfere with normal embryo development, and adverse outcomes could be expected, depending on the type and extension of the DNA lesions (Ahmadi and Ng, 1999; Aitken and Krausz, 2001). At present, it is not clear how styrene could cause DNA strand breaks in this cell type. There is little information on the presence of styrene metabolite levels in various organs, particularly in the reproductive ones (Brown *et al.*, 2000). Direct exposure of reproductive organs via skin absorption of styrene cannot be excluded. The presence of the haematotesticular barrier, the antioxidant capacity of both sperm and seminal plasma, and a high degree of chromatin packaging protect male germ cells, even though they lack an active DNA repair system during the maturation and differentiation stages (Lewis, 1999). However, the exposure of sperm to

excessive levels of reactive oxygen species (ROS) has the capacity to damage lipids in the sperm plasma membrane and also to oxidize nuclear DNA (Sharma *et al.*, 1999). Since DNA breakage represents non-specific damage, other mechanisms leading to this kind of DNA damage have to be considered. In particular, oxidative stress is known to cause DNA fragmentation and the oxidative modification of DNA bases, such as 8-hydroxy-deoxyguanosine (8-OH-dG) (Aitken and Krausz, 2001). Styrene-7,8-oxide, the main intermediate and highly reactive metabolite of styrene, may directly produce protein, RNA and DNA adducts. Together with an enzyme deficiency in detoxification enzymes, these adducts could cause oxidative stress by producing ROS, due to the imbalance between oxidant and antioxidant systems, which can involve very reactive hydroxyl radicals (Marczynski *et al.*, 2000). Following styrene exposure, oxidative DNA damage may be the result of the production of ROS: this may contribute to the elevated levels of DNA strand breaks observed in white blood cells of styrene-exposed workers, although increased DNA strand break levels, detected using the Comet assay, correlated with styrene air concentration at the workplace and with urinary MA, when both parameters of exposure were analysed on the day of sampling (Vodicka *et al.*, 1999). Moreover, in repeated samplings significant correlation was found between DNA damage and styrene-specific O⁶-guanine DNA adducts (Vodicka *et al.*, 1995, 1999). Styrene oxide ability to induce single-strand breaks and alkali labile sites was also detected *in vitro* in both somatic cells (Vodicka *et al.*, 1996) and in isolated rat and human testicular cells by Bjorge and colleagues in a comparative study assessing DNA damage produced by a number of different chemicals (Bjorge *et al.*, 1996). Thus, the increase in DNA strand breaks observed in the sperm of workers employed in the reinforced plastics industry in our study may be either the result of styrene oxide alkylation or due to the oxidative stress associated with the metabolism of styrene. An implementation of the analysis of styrene-specific DNA adducts (SO-1-adenines, SO-7-guanines) in sperm cells from styrene-exposed workers may cast some light on the mechanism of styrene-induced DNA damage.

The Comet assay, a fast and sensitive technique, is increasingly used in molecular epidemiology and in studies on DNA repair. In particular, the method is devoted to the assessment of primary DNA or oxidative DNA damage, in several cell types of subjects environmentally or occupationally exposed to mutagens/carcinogens or affected by various pathological conditions (Kassie *et al.*, 2000). Recently, clinical applications of the Comet assay have also addressed DNA integrity in male germ cells of fertile and infertile men within the context of assisted reproduction (McKelvey-Martin *et al.*, 1997; Donnelly *et al.*, 2001) or sperm DNA damage after treatment with chemotherapy (Chatterjee *et al.*, 2000). A significant negative relationship between semen quality analysis, particularly sperm concentration, and sperm DNA fragmentation in patients with infertility problems has been reported (Irvine *et al.*, 2000). In the present study, subjects with a reduction in sperm concentration also exhibited high levels of DNA damage in the same cells. In our hands, the Comet assay proved to be sensitive in detecting effects of occupational exposure to styrene in male

germ cells, whereas sperm concentration and morphology did not differ between exposed and unexposed subjects. In our study, DNA fragmentation increased positively with chronological age. Conflicting results have been reported on the effect of chronological age, among other confounding factors, on the results of the Comet assay in biomonitoring studies conducted in somatic cells (Moller *et al.*, 2000). Very recently a positive correlation between sperm DNA damage detected by the comet assay and age has been observed in 60 randomly selected men undergoing IVF treatment (Morris *et al.*, 2002).

The present study suggests that exposure to styrene at the workplace may result in DNA fragmentation in germ cells of male workers. However, there was no correlation between the DNA damage in sperm cells and urinary MA, probably due to the fact that semen samples and urinary samples were not collected on the same day. In addition, contact of styrene with the skin and subsequent absorption might also have contributed to the DNA damage. At the moment it is uncertain in which stage of spermatogenesis the DNA damage is being induced and very scarce information is available on DNA repair (i.e. the persistence of the DNA damage is virtually unknown). Without this essential information it is difficult to relate acute styrene exposure (reflected by the level of urinary MA) to the extent of DNA damage in sperm cells and to establish when DNA might have been impaired during the 72 day period of spermatogenesis.

Our investigation has revealed the potential of styrene exposure to induce DNA damage in sperm cells. In order to understand the mechanisms inflicting DNA damage in sperm cells, the following issues have to be addressed in future studies: (i) the effective exposure of sperm cells to styrene (particularly relevant are routes of exposure, relationships between internal and external exposure parameters, role of duration of exposure and possible cumulative effects); (ii) the kinetics of DNA damage formation and removal (particularly the role of DNA repair); and (iii) the nature of DNA damage (styrene-specific DNA adducts or 8-OH-dG adducts).

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References

- Ahmadi, A. and Ng, S.C. (1999) Developmental capacity of damaged spermatozoa. *Hum. Reprod.*, **14**, 2279–2285.
- Aitken, R.J. and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction*, **122**, 497–506.
- Alexander, B.H., Checkoway, H., van Netten, C., Muller, C.H., Ewers, T.G., Kaufman, J.D., Mueller, B.A., Vaughan, T.L. and Faustman, E.M. (1996) Semen quality of men employed at a lead smelter. *Occup. Environ. Med.*, **53**, 411–416.

- American Conference of Governmental Industrial Hygienists (2000) *Styrene, Monomer in ACGIH Documentation of the Threshold Limit Values and Biological Exposure Indices*. Science Group ACGIH, Cincinnati, USA, 2000.
- Bigelow, P.L., Jarrell, J., Young, M.R., Keefe, T.J. and Love, E.J. (1998) Association of semen quality and occupational factors: comparison of case-control analysis and analysis of continuous variables. *Fertil. Steril.*, **69**, 11–18.
- Bjorge, C., Brunborg, G., Wiger, R., Holme, J.A., Scholz, T., Dybing, E. and Soderlund, E.J. (1996) A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. *Reprod. Toxicol.*, **10**, 509–519.
- Bond, J.A. (1989) Review of the toxicology of styrene. *Crit. Rev. Toxicol.*, **19**, 227–249.
- Brown, N.A., Lamb, C.J., Brown, S.M. and Neal, B.H. (2000) A review of the developmental and reproductive toxicity of styrene. *Regul. Toxicol. Pharmacol.*, **32**, 228–247.
- Chatterjee, R., Haines, G.A., Perera, D.M.D., Goldstone, A. and Morris, I.D. (2000) Testicular and sperm DNA damage after treatment with fludarabine for chronic lymphocytic leukaemia. *Hum. Reprod.*, **15**, 762–766.
- Chia, S.E., Ong, C., Tsakok, M.F.H. and Ho, A. (1996) Semen parameters in workers exposed to trichloroethylene. *Reprod. Toxicol.*, **10**, 295–299.
- De Kretser, D.M. (1997) Male infertility. *Lancet*, **349**, 787–790.
- Donnelly, E.T., Stelle, E.K., McClure, N. and Lewis, S.E. (2001) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum. Reprod.*, **16**, 1191–1199.
- Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) The comet assay: a comprehensive review. *Mutat. Res.*, **339**, 37–59.
- Figa-Talamanca, I., Dell'Orco, V., Pupi, A., Dondero, F., Gandini, L., Lenzi, A., Lombardo, F., Scavalli, P. and Mancini, G. (1992) Fertility and semen quality analysis of workers exposed to high temperatures in the ceramics industry. *Reprod. Toxicol.*, **6**, 517–523.
- Friedler, G. (1996) Paternal exposures: impact on reproductive and developmental outcome: an overview. *Pharmacol. Biochem. Behav.*, **55**, 691–700.
- International Agency for Research on Cancer (1994) *Some Industrial Chemicals, IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, N°60*. IARC, Lyon.
- Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A. and Aitken, R.J. (2000) DNA integrity in human spermatozoa: relationship with semen quality. *J. Androl.*, **21**, 33–44.
- Jelnes, J.E. (1988) Semen quality in workers producing reinforced plastic. *Reprod. Toxicol.*, **2**, 209–212.
- Kassie, F., Parzefall, W. and Knasmüller, S. (2000) Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. *Mutat. Res.*, **463**, 13–31.
- Kolstad, H.A., Bonde, J.P.E., Spano, M., Giwercman, A., Zschiesche, W., Kaae, D. and Roeleveld, N. (1999) Sperm chromatin structure and semen quality following occupational styrene exposure. *Scand. J. Work Environ. Health*, **25**, 70–73.
- Lauwerys, R.R. and Hoet, P. (1993) *Industrial Chemical Exposure: Guidelines for Biological Monitoring*, 2nd edn. Lewis Publishers, CRC Press, Boca Raton, FL.
- Lewis, S.E. (1999) Life cycle of the mammalian germ cell: implication for spontaneous mutation frequencies. *Teratology*, **59**, 205–209.
- Marczynski, B., Peel, M. and Baur, X. (2000) New aspects in genotoxic risk assessment of styrene exposure—a working hypothesis. *Med. Hypoth.*, **54**, 619–623.
- Martin, R.H., Ernst, S., Rademaker, A., Barclay, L., Ko, E. and Summers, N. (1999) Analysis of sperm chromosome complements before, during and after chemotherapy. *Cancer Genet. Cytogenet.*, **108**, 133–136.
- McKelvey-Martin, V.J., Melia, N., Walsh, I.K., Johnston, S.R., Hughes, C.M., Lewis, S.E.M. and Thompson, W. (1997) Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). human bladder washings and transitional cell carcinoma of the bladder; and (2). human sperm and male infertility. *Mutat. Res.*, **375**, 93–104.
- Mocarelli, P., Gerthoux, P.M., Ferrari, E., Patterson, D.G., Kieszak, S.M., Brambilla, P., Vincoli, N., Signorini, S., Tramacere, P. and Carreri, V. (2000) Paternal concentrations of dioxin and sex ratio of offspring. *Lancet*, **355**, 1858–1863.
- Moller, P., Knudsen, L.E., Loft, S. and Wallin, H. (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 1005–1015.
- Morris, I.D., Iltot, S., Dixon, L. and Brison, D.R. (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum. Reprod.*, **17**, 990–998.
- Oliva, A., Spira, A. and Multigner, L. (2001) Contribution of environmental factors to the risk of male infertility. *Hum. Reprod.*, **16**, 1768–1776.
- Olive, P.L. (1999) DNA damage and repair in individual cells: applications of the comet assay in radiobiology. *Int. J. Radiat. Biol.*, **75**, 395–405.
- Poggi, G., Giusiani, M., Palagi, U., Paggiaro, P.L., Loi, A.M., Dazzi, F., Siclari, C. and Baschieri, L. (1982) High-performance liquid chromatography for the quantitative determination of the urinary metabolites of toluene, xylene and styrene. *Int. Arch. Occup. Environ. Health*, **50**, 25–31.
- Robbins, W.A., Vine, M.F., Young Truong, K. and Everson, R.B. (1997) Use of fluorescence *in situ* hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. *Environ. Mol. Mutagen.*, **30**, 175–183.
- Sharma, R.K., Pasqualotto, F.F., Nelson, D.R., Thomas, A.J. and Agarwal, A. (1999) The reactive oxygen species – total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum. Reprod.*, **14**, 2801–2807.
- Symanski, E., Bergamaschi, E. and Mutti, A. (2001) Inter- and intra-individual sources of variation in levels of urinary styrene metabolites. *Int. Arch. Occup. Environ. Health*, **74**, 336–344.
- Takao, T., Nanamiya, W., Nazarloo, H.P., Asaba, K. and Hashimoto, K. (2000) Possible reproductive toxicity of styrene in peripubertal male mice. *Endocr. J.*, **47**, 343–347.
- Tates, A.D., Grummt, T., van Dam, F.J., de Zwart, F., Kasper, F.J., Rothe, R., Stirn, H., Zwinderman, A.H. and Natarajan, A.T. (1994) Measurement of frequencies of HPRT mutants, chromosomal aberrations, micronuclei, sister-chromatid exchanges and cells with high frequencies of SCEs in styrene/dichloromethane-exposed workers. *Mutat. Res.*, **313**, 249–262.
- Vodicka, P., Bastlova, T., Vodickova, L., Peterkova, K., Lambert, B. and Hemminki, K. (1995) Biomarkers of styrene exposure in lamination workers: levels of O⁶-guanine DNA adducts, DNA strand breaks and mutant frequencies in the hypoxanthine guanine phosphoribosyltransferase gene in T-lymphocytes. *Carcinogenesis*, **16**, 1473–1481.
- Vodicka, P., Stetina, R., Kumar, R., Plna, K. and Hemminki, K. (1996) 7-Alkylguanine adducts of styrene oxide determined by ³²P-postlabelling in DNA and human embryonal lung fibroblasts (HEL). *Carcinogenesis*, **17**, 801–808.
- Vodicka, P., Tvrdik, T., Osterman-Golkar, S., Vodickova, L., Peterkova, K., Soucek, P., Sarmanova, J., Farmer, P.B., Granath, F., Lambert, B. et al. (1999) An evaluation of styrene genotoxicity using several biomarkers in a 3-year follow-up study of hand-lamination workers. *Mutat. Res.*, **445**, 205–224.
- Vodicka, P., Koskinen, M., Arand, M., Oesch, F. and Hemminki, K. (2002) Spectrum of styrene-induced DNA adducts: the relationship to other biomarkers and prospects in human biomonitoring. *Mutat. Res.*, **511**, 239–254.
- Wang, S.L., Wang, X.R., Chia, S.E., Shen, H.M., Song, L., Xing, H.X., Chen, H.Y. and Ong, C.N. (2001) A study on occupational exposure to petrochemicals and smoking on seminal quality. *J. Androl.*, **22**, 73–78.
- Whorton, D., Krauss, K.M., Marshall, S. and Milby, T.H. (1977) Infertility in male pesticide workers. *Lancet*, **ii**, 1259–1261.
- World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*, 3rd edn. Cambridge University Press, Cambridge.
- World Health Organization (1996) *WHO Biological Monitoring of Chemical Exposure in the Workplace, Guidelines*, vol. 1. WHO, Geneva.
- Wyrobek, A.J. (1993) Methods and concepts in detecting abnormal reproductive outcomes of paternal origin. *Reprod. Toxicol.*, **7**, 3–16.

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