Cytogenetic Markers, DNA Single-Strand Breaks, Urinary Metabolites, and DNA Repair Rates in Styrene-Exposed Lamination Workers

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The effect of occupational exposure to styrene on frequencies of chromosomal aberrations and binucleated cells with micronuclei and on single-strand break levels in peripheral blood lymphocytes was studied in 86 reinforced plastic workers and 42 control individuals (including 16 maintenance workers with intermittent, low-dose exposure). In these individuals, the irradiation-specific DNA repair rates and the repair rates of 8-oxoguanines were investigated. We assessed the exposure by measuring the concentrations of styrene in air and in blood and of mandelic acid, phenylglyoxylic acid, 4-vinyl phenol conjugates and regioisomeric phenyl hydroxyethyl mercapturic acids in urine. All these parameters correlated with one another. No clear relationship was found between the styrene exposure and the frequencies of chromosomal aberrations. Binucleated cells with micronuclei were moderately related to the parameters of styrene exposure. We found a negative correlation between all exposure parameters and single-strand breaks. The positive correlation between exposure parameters and DNA repair rates suggests that particular DNA repair pathways may be induced by styrene exposure. Key words: DNA repair rates, genotoxicity, styrene exposure, urinary metabolites. Environ Health Perspect 112:867–871 (2004). doi:10.1289/ehp.6849 available via http://dx.doi.org/ [Online 12 February 2004]

Styrene is used widely in the production of plastics, synthetic rubber, and polyester resins and occurs as an environmental contaminant, present in small amounts in food items, tobacco smoke, and engine exhausts. Occupational exposure in hand lamination work in the reinforced plastic industry may entail a daily intake of gram quantities of styrene via inhalation [International Agency for Research on Cancer (IARC) 2002].

Many genetic toxicology assays are positive for styrene [reviewed by World Health Organization (WHO) 2001]. IARC has classified styrene as a possible human carcinogen (group 2B), with limited evidence for carcinogenicity in humans and in experimental animals, whereas its main intermediary metabolite, styrene-7,8-oxide (StO), has been classified as a probable human carcinogen (group 2A; IARC 1994). A recent hypothesis has postulated that oxidative stress may arise as a result of epoxide hydrolase to phenylethylene glycol, genases, is primarily detoxified by microsomal enzymes. Thus far, little information is available on the possible effect of styrene exposure on DNA repair rates. An early study indicated that styrene exposure, both in vivo and in vitro, increased unscheduled DNA synthesis induced in human mononuclear leukocytes by N-acetoxy-2-acetylaminofluorene but not by ultraviolet radiation (Pero et al. 1982). The authors concluded that styrene exposure does not alter the efficiency of DNA repair synthesis but only the susceptibility of DNA toward damages from chemical mutagens that depend on cellular metabolism. However, preliminary studies using the comet assay have suggested that the capacity of lymphocytes to remove both γ-ray–induced DNA damage and oxidative DNA damage is increased in styrene-exposed workers (Vodicka et al. 2003).

In the present study, we examined the effect of occupational exposure to styrene on the frequency of chromosomal aberrations (CAs) and binucleated cells (BNs) with micronuclei (MN) and on the level of SSBs and SSB endonuclease III (Endo III) sites in peripheral lymphocytes. An innovative aspect was an investigation of the irradiation-specific DNA repair rates and the repair rates of 8-oxoguanines. Whether the level of various biomarkers is modulated by polymorphisms in DNA repair genes as well as by individual DNA repair rates (particularly those related to styrene-induced DNA lesions) in styrene-exposed subjects has to be addressed in the future investigation.

The discrepancy between an excessive, long-lasting occupational exposure to styrene and relatively low levels of measured biomarkers is increased in styrene-exposed workers (Koskinen et al. 2001; Vodicka et al. 2003) and inconclusive outcomes from cancer epidemiology studies (Kogevinas et al. 1993) indicate that various mechanisms connected with styrene biotransformation and DNA repair may be of importance. Recent studies have reported that GSTM1 genotype along with GSTT1 genotype may affect the genotoxicity of styrene in human lymphocytes in vitro (Bernardini et al. 2002) and urinary excretion of PHEMAs in styrene-exposed workers (Haufroid et al. 2002). In addition, genetic polymorphisms of biotransformation enzymes have been suggested to modulate the genotoxicity in styrene-exposed workers (Vodicka et al. 2001), although no strict conclusions can yet be drawn.
Materials and Methods

Subjects. The styrene-exposed group consisted of 86 workers employed in three plants (designated A, B, and C) located in the same area. The control group consisted of 26 employees of the Regional Hygienic Station (external control; EC) and 16 maintenance workers from plant B (plant control; PC). The design of the study was approved by the local ethical committee, and all participants provided their informed consent. The sampling of biologic material was carried out according to the Declaration in Helsinki (World Medical Association Declaration of Helsinki 1964). The exposed and control workers had similar socioeconomic background; the exposed subjects were, in comparison with the controls, on average 8.7 years younger, included more men (71 vs. 52%) and smokers (51 vs. 19%), and had smoked about twice as many cigarettes during their lifetime (Table 1). These differences were taken into account in the statistical analyses.

Styrene exposure in workplace air and in blood. The concentration of styrene in the workplace air was determined by personal dosimeters the day of the sampling (Vodicka et al. 1995). The determination of styrene concentration in blood has been described previously (Vodicka et al. 1995, 2001).

Styrene-specific urinary metabolites. An aliquot of urine was collected at the end of shift concomitantly with other samplings and used for the determination of the styrene-specific urinary metabolites MA, PGA, 4-VPT, and regioisomeric PHEMAs. The methodologies have been described previously for MA and PGA (Symanski et al. 2001), for 4-VPT (Manini et al. 2002), and for the PHEMAs diastereomeric N-acetyl-S-(1-phenyl-2-hydroxyethyl)-cysteine (M1RR), (M1RS), and N-acetyl-S-(2-phenyl-2-hydroxyethyl)-cysteine (M2) (Ghittori et al. 1997).

Determination of SSBs and SSB Endo III sites. SSBs, reflecting DNA damage induced by alkylation, were detected by a modified single-cell gel electrophoresis (comet) assay (Collins et al. 1996). SSB Endo III sites, reflecting abasic sites and oxopyrimidines, were evaluated by a modified version of comet assay with Endo III treatment (Collins et al. 1996) and are expressed as net values after the subtraction of SSBs.

Cytogenetic analyses. The techniques used for the determination of CA (Valjus et al. 1993) and MN (Migliore et al. 1999) have been described previously. One hundred metaphases per sample for CA and 1,000 BNs/sample for MN were scored from coded slides.

DNA repair rates. Peripheral blood lymphocytes isolated using Ficol gradient were used to test individual DNA repair capacity as described previously (Alapetite et al. 1999; Vodicka et al. 2003). Briefly, cells embedded in agarose on slides were irradiated with 5 gray (Gy) of γ-rays (0.42 Gy/min) and either lysed immediately or incubated at 37°C for 40 min before the lysis. The DNA breaks induced by γ-rays are repaired according to the individual repair capacity, and the results are expressed as amount of repaired SSBs. Simultaneously with the analysis in human lymphocytes, irradiation-induced SSBs and remaining SSBs in various intervals were assayed for in human fibroblasts. This test confirmed that SSB removal is measured and the time interval is appropriate (Figure 1).

In addition, the individual capacity of peripheral lymphocytes to extract repair 8-oxoguanine, known to be removed from DNA by a specific glycosylase (oxoguanine glycosylase; OGG1), was determined as previously described (Collins et al. 2001). Briefly, HeLa cells were pretreated with the photosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland), irradiated with a fluorescent lamp to induce 8-oxoguanine, incubated with cell extracts prepared from lymphocytes of each subject, and analyzed by the comet assay. The level of SSBs reflects the removal of 8-oxoguanine from HeLa cell DNA by the lymphocyte extract.

Statistical analyses. Statistical calculations were performed using Statgraphics, version 7 (Manugistics, Inc., Cambridge, MA, USA). The data were not normally distributed, and nonparametric tests were used: Mann-Whitney U-test for testing significant differences between groups, Spearman correlation analysis for estimation of the correlation between parameters, and the Kruskal-Wallis test for the associations between biomarkers.

All analyses were performed with coded samples.

Results

Styrene exposure. The mean styrene concentration in the workplace air, determined by personal dosimeters in three plants, was 81.3 ± 56.3 mg/m³; the highest mean concentration was in plant A, followed by plant C and plant B (Table 1).

Styrene concentration in blood was, on average, 0.56 ± 0.43 mg/L in the exposed group and 0.07 ± 0.06 mg/L in the controls (Table 1). In the control group, styrene concentrations in blood were exclusively recorded among the plant controls, suggesting that low-level, intermittent exposure to styrene

Table 1. Characteristics of the studied population and indicators of exposure to styrene.

| Characteristics                  | All exposed (n = 86) | Plant A (n = 35) | Plant B (n = 31) | Plant C (n = 20) | Plant controls (n = 16) | External controls (n = 26) |
|----------------------------------|---------------------|-----------------|-----------------|-----------------|------------------------|--------------------------|
| Age (years [mean ± SD])          | 36.5 ± 12.0         | 36.8 ± 12.6     | 38.0 ± 12.6     | 33.7 ± 9.5      | 45.8 ± 7.6              | 42.8 ± 8.2               |
| Sex (F/M)                        | 25/61               | 15/20           | 0/31            | 10/10           | 0/16                   | 20/6                     |
| Smoking habit                    | 44 S/42 NS          | 15 S/20 NS      | 16 S/15 NS      | 13 S/7 NS       | 2 S/14 NS               | 6 S/20 NS                |
| Lifetime no. of cigarettes       | 80,711 ± 76,529     | 58,853 ± 64,008 | 114,850 ± 96,236 | 62,761 ± 45,420 | 127,750 ± 5,162         | 85,600 ± 62,591          |
| Years of employment (mean ± SD)  | 4.0 ± 3.1           | 3.4 ± 3.3       | 5.6 ± 3.4       | 2.5 ± 1.9       | ND                     | ND                       |
| Workplace styrene               | 81.3 ± 56.3         | 112.4 ± 57.5    | 47.1 ± 41.9     | 82.4 ± 43.9     | ND                     | ND                       |
| Exposure [mg/m³ [mean ± SD)]     | 0.56 ± 0.43         | 0.71 ± 0.47     | 0.40 ± 0.31     | 0.50 ± 0.44     | 0.07 ± 0.06             | ND                       |
| Styrene levels in blood [mg/L (mean ± SD)] | 0.56 ± 0.43 | 0.71 ± 0.47 | 0.40 ± 0.31 | 0.50 ± 0.44 | 0.07 ± 0.06 | ND |
| MA + PGA levels [mg/g creatinine (mean ± SD)] | 496.9 ± 402.2 | 797.5 ± 418.9 | 269.9 ± 180.0 | 307.8 ± 271.9 | 42.1 ± 15.5 | ND |
| 4-VPT levels [mg/g creatinine (mean ± SD)] | 5.64 ± 4.82 | 7.82 ± 5.70 | 3.38 ± 2.23 | 5.63 ± 4.71 | 0.39 ± 0.39 | ND |
| PHEMAs levels [mg/g creatinine (mean ± SD)] | M1 [RR + RS] | 1.75 ± 4.11 | 2.09 ± 3.05 | 0.40 ± 0.64 | 3.05 ± 6.61 | ND |
|                                  | M2                  | 1.37 ± 3.22     | 1.46 ± 1.78     | 0.33 ± 0.40     | 3.14 ± 6.32              | ND                       |

Abbreviations: F, female; M, male; ND, not determined; NS, nonsmokers; S, smokers.
might occur among the maintenance workers. Although these workers were not directly involved in the styrene processing (as declared also in questionnaires), the low-level styrene exposure was further supported by the levels of urinary metabolites. In exposed workers, the concentrations of styrene in the air were correlated with those in blood ($R = 0.817$, $p < 0.001$).

**Styrene-specific urinary metabolites.** The styrene metabolites determined in the urine of laminators followed the levels shown by external exposure parameters (Table 1). The mean levels of MA, PGA, and 4-VPT in the laminators were more than 10 times higher than those of the plant controls. In the exposed workers, urinary concentrations of various PHEMAs were $1.8 \pm 4.1$ mg/g creatinine for M1RR and M1RS, and $1.4 \pm 3.2$ mg/g creatinine for M2. PHEMAs were not determined in the control groups. All urinary metabolites in all exposed individuals and in workers from each particular plant correlated strongly with each other as well as with the concentrations of styrene in the air and in blood (data not shown).

**Cytogenetic markers.** CA frequencies are presented in Table 2. The highest mean CA frequency was in the EC group ($3.2 \pm 2.0$), and the lowest was in the plant controls ($1.7 \pm 0.9$; $p = 0.042$). Workers from plant A, exposed to the highest styrene concentration (mean, $112$ mg/$m^3$), exhibited the second highest CA frequency ($2.5 \pm 1.6$) but did not differ significantly from the other groups. Results on chromatic-type aberrations (with and without gaps) and on chromosome breaks followed the same pattern as the frequency of total aberrant cells (Table 2) and did not correlate with any marker of styrene exposure. CA frequencies correlated positively with age ($R = 0.223$, $p = 0.014$); this correlation was more pronounced for chromosome-type CA ($R = 0.228$, $p = 0.012$). No influence of other confounders on CA frequencies was recorded.

The highest mean BN MN frequency was recorded among the workers from plant A and the ECs, and the lowest was in plant controls (Table 2). An association between BN MN frequency and styrene exposure ($p = 0.001$ for styrene in air and $p = 0.001$ for styrene in blood) was found. In addition, BN MN frequency correlated with MA, PGA, and 4-VPT (data not shown). Significantly elevated BN MN frequencies were found in individuals with a higher index for cumulative exposure (styrene concentration in air multiplied by duration of exposure) than in those with a lower index ($17.6 \pm 7.2$ vs. $13.9 \pm 6.2$, $p = 0.017$; Figure 2). BN MN frequencies significantly increased with age ($R = 0.231$, $p = 0.013$) and were higher in women than in men ($F = 25.7$, $p < 0.001$).

SSBs in DNA and SSB Endo III sites. The lower mean level of SSBs was recorded in plant A in comparison with both plant and ECs (Table 2). In fact, SSBs negatively correlated with most markers of styrene exposure ($R = -0.350$, $p = 0.007$ for styrene in blood; $R = -0.402$, $p = 0.01$ for MA; $R = -0.403$, $p = 0.001$ for PGA; $R = -0.375$, $p = 0.003$ for 4-VPT). Decrease of SSBs with increasing concentration of styrene in air is illustrated in Figure 3A. SSB levels were not affected by any confounder recorded.

The mean level of SSB Endo III sites were not significantly different among different groups of exposed subjects and in comparison with the control individuals (Table 2). The levels of SSB Endo III sites did not correlate with any of the exposure markers, including urinary metabolites.

**DNA repair rates.** As shown in Figure 1, most SSBs induced by γ-rays are rapidly removed from DNA with an approximate half-life of 15–20 min. There is still residual DNA damage, which may be unrepaired double-strand breaks (DSBs).

Individual capacity to repair SSBs induced by γ-rays was significantly lower in the ECs, compared with all exposed groups ($p = 0.023$ vs. plant A, $p = 0.016$ vs. plant B, $p = 0.001$ vs. plant C; Table 2). Irradiation-specific DNA repair rates were associated with styrene concentration in air (Figure 3B), correlated significantly with styrene in blood ($R = 0.308$, $p = 0.031$), and showed a tendency to decrease with increasing age ($R = -0.204$, $p = 0.095$).

The capacity of lymphocytes to incise 8-oxoguanine in the different groups is shown in Table 2. The only significant (> 2-fold) increase in the DNA repair was recorded among workers from plant A ($p = 0.0006$ vs. plant B, $p = 0.0007$ vs. plant C, $p = 0.0003$ vs. plant controls, $p = 0.015$ vs. ECs). Repair capacity to remove oxidative DNA damage increased with increasing styrene concentration in air (Figure 3C) and correlated significantly with styrene in blood ($R = 0.371$, $p = 0.004$) and with urinary metabolites ($R = 0.414$, $p = 0.002$ for MA; $R = 0.428$, $p = 0.001$ for PGA; $R = 0.336$, $p = 0.010$ for 4-VPT).

**Discussion**

In this study, we examined the levels of specific urinary metabolites and chromosome and DNA damage in relation to DNA repair rates in styrene-exposed workers.

**Exposure assessment.** The strong correlations observed between measures of external and internal styrene exposure and styrene-specific urinary metabolites have been well documented (Vodicka et al. 1999). As an interesting new finding concerning the metabolites, the urinary excretion of both 4-vinyl phenol conjugates 4-vinyl phenol glucuronide and 4-vinyl phenol sulfate was exposure dependent, indicating that 3,4-arene oxidation...
takes place in styrene metabolism in humans and may contribute to the genotoxic burden. Although this metabolic pathway has been previously postulated (Paffili et al. 1981), the biologic relevance of styrene-3,4-oxide, for example, in terms of DNA adduct formation has yet to be investigated. Exposure-dependent excretion of diastereomeric PHEMAs in workers provides only limited information, because conjugation of StO with glutathione represents a minor metabolic pathway (< 1% of the total urinary metabolites), and is highly dependent on GSTM1 polymorphism. GSTM1-positive individuals have been shown to excrete five to six times more PHEMA than do GSTM1-negative subjects (De Palma et al. 2001).

Cytogetic markers. In contrast to previous findings (Artuso et al. 1995; Somorovska et al. 1999), we did not observe any effect of styrene exposure on CA frequencies; nor were chromatid-type aberrations, previously shown to be indicative of styrene treatment (Jantunen et al. 1986), increased in the exposed subjects. The BN MN frequencies were associated with the concentrations of styrene in the air and in blood and of urinary metabolites and with cumulative exposure index, suggesting an effect of the occupational exposure on MN formation, after adjustment for known confounders (age and sex). However, the differences in the mean BN MN frequency between the exposed group and ECs were negligible, whereas the plant control group showed the lowest BN MN frequency. Remarkably, a higher BN MN frequency in the EC group may be due to the known confounders affecting this end point: age and sex (Fenech 1998); indeed, the EC group consisted mainly of women with higher mean age. Similar considerations may be plausible for frequencies of CAs.

SSBs and SSB Endo III sites. SSBs in DNA were significantly lower in exposed workers than in those unexposed and correlated inversely with parameters of internal exposure. This is in striking contrast to previous studies (Vodicka et al. 1999), where an exposure-related increase in SSBs was found and SSBs correlated significantly with O6-styrene–guanine DNA adducts (Vodicka et al. 1995) and CA (Somorovska et al. 1999). This discrepancy may be caused by differences among the populations examined: The previous consisted of individuals exposed for 14 years on average, whereas workers in the present study were exposed to styrene for less than 4 years. On the other hand, in the present study the relatively lower SSB levels in exposed workers were associated with more efficient DNA repair capacities, whereas in previous reports DNA repair rates were not addressed.

SSB Endo III sites, reflecting either abasic sites or oxoprimidines, were almost identical in both the exposed and control groups, but in contrast to our previous study (Somorovska et al. 1999), we recorded this type of DNA damage at clearly distinguishable levels. Because oxidative DNA damage may arise because of various intrinsic and extrinsic factors and SSB Endo III sites represent a nonspecific marker, it is difficult to pinpoint the origin of this DNA damage in the present population. The role of oxidative stress related to styrene exposure may be a contributing factor, as indicated by increased levels of 8-hydroxyguanine DNA adducts among workers exposed to styrene (Marczynski et al. 2000).

DNA repair rates. To screen individual DNA repair rates, we employed a modified comet assay on irradiated human lymphocytes. The comet assay is a useful tool for the DNA damage and repair assessment (Dikomey et al. 2000; Popanda et al. 2003), but its version based on alkaline denaturation does not allow discrimination of simultaneously induced SSBs and DSBs. Irradiation-induced (γ-rays) DNA damage consists of mainly SSBs (90%), whereas DSBs account for 10% (Sakai and Okada 1984). Because most of the DNA damage induced by irradiation is repaired very quickly, this repair pathway may be attributable to base excision repair. In fact, the irradiation-related DSBs are repaired with half-life of more than 2 hr (Dikomey and Franke 1986) via nonhomologous recombination repair (Natarajan 2002). We investigated individual capacities to repair SSBs induced by irradiation. On the other hand, SSBs were also analyzed as an indicator of DNA damage without any information on their specificity and origin. Despite the limitations in specificity, the association between the actual levels of SSBs and the capacities of their repair is important.

Capacity to repair irradiation-specific DNA damage was significantly higher in exposed workers than in unexposed or moderately exposed subjects. The apparent increase in DNA repair rates related to styrene exposure may reflect effective activation of the DNA repair machinery. It remains to be resolved whether DNA repair rates are truly induced, whether a threshold exists in this induction, and whether long-term exposure could exhaust the induction.

An increased capacity of lymphocytes to incise 8-oxoguanine was recorded among highly exposed workers. Significant association between both internal and external exposure parameters and repair capacity to remove oxidative DNA damage suggests a possible role of oxidative stress in styrene-related genotoxicity, as previously postulated.

However, until we address combinations of various exposure parameters and multiple markers of early effect, interpretations should be cautious, and the complexity of the biologic system has to be considered in order to prevent simple mechanistic extrapolation. In styrene-exposed workers, genotoxic effects may depend on the extent of exposure, duration of exposure, and individual susceptibility in several biotransformation and DNA repair genes, as well as adaptation processes. As suggested by the present pilot study, the role of individual DNA repair rates deserves further investigation.

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Environmental Health Perspectives • VOLUME 112 • NUMBER 8 • June 2004

Article | Individual response to styrene exposure

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