Biological Monitoring of Workers Exposed to \(N\)-Nitrosodiethanolamine in the Metal Industry

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Workers in the metal industry may be exposed to \(N\)-nitrosamines because these potentially genotoxic/carcinogenic substances have been detected in cutting and grinding fluids (1–4). \(N\)-Nitrosodiethanolamine (NDELA) is the most common \(N\)-nitrosamine found in cutting fluids formulated with nitrite and ethanolamine (diethanolamine is present as an impurity) (5). NDELA is a strong animal carcinogen (6–8), a mutagen in the Ames test after activation with alcohol dehydrogenase (9,10), and a potent inducer of DNA damage in primary hepatocytes in vitro (11). Because animal studies have shown that 60–90% of NDELA is excreted in urine, analysis of NDELA urine excretion from workers in the metal industries has been suggested for biological monitoring of the occupational exposure to this nitrosamine (2,3).

Using an integrated environmental/biological monitoring approach previously applied to different occupational exposures by our group (12), we analyzed several cutting fluids sampled in some metal working factories in Central Italy and regularly used cutting oils (exposed group), which had been previously analyzed for nitrite and NDELA content and mutagenicity (13). The workers were divided into two groups according to the NDELA content of the cutting fluids used: a high-exposure group \((n = 25)\) using cutting fluids with NDELA content \(>25\) mg/L, and a low-exposure group \((n = 37)\) using fluids with NDELA content \(<5\) mg/L. We recruited as a control group male subjects who worked in the same factories as office personnel \((n = 37)\) and were not exposed to cutting fluids or other chemicals.

Data on smoking status, medicine consumption, and alcohol and coffee drinking were collected by means of a short, self-administered questionnaire. All the ex-smokers had stopped smoking at least 6 months before sample collection. Since some drugs influence the hepatic activity of certain enzymes, medicine consumption was taken into account for the analysis of urinary DGA as a potential confounder.

**Urine sampling.** Urine samples from exposed and control subjects were collected on Thursday (i.e., after 3 working days) before working hours and 4 hr after the beginning of the shift. The samples were kept cool during transport and then stored at -20°C until assayed.

**Urine NDELA determination.** NDELA content was determined in 15-ml urine samples, according to the method of Spiegelhalder et al. (2.3). Nitrite was destroyed with sulfamic acid. NDELA was extracted using Kieselguhr extraction columns and ethyl formate containing 2% methanol. Extracts were evaporated to dryness under a stream of nitrogen, and the residue reacted with 0.3 ml of a silating agent \((N\)-methyl-\(N\)-trimethylsilylhexafluoro-2-propanamide\) at 80°C for 2 hr. NDELA analysis was carried out by gas chromatography/chemiluminescence detection (TEA 502). The gas chromatograph conditions were: injector, 200°C; on-column injection; column, 0.635 cm o.d., 0.2 cm i.d. × 140 cm silanized borosilica glass filled with 6% OV275 on Vosapher A2 (Merck, Germany); oven, initial temperature 110°C, 5 min, final temperature programme 10°C/min, final temperature 220°C, 5 min.

We identified NDELA by comparing retention time with authentic reference material in solution (500 \(\mu g/mL\), purity > 99.9%). Quantification was based on comparison of peak areas. Under the conditions used, no interfering peaks were observed. Recovery was determined using spiked blank urines and was 80–87%. Results were not corrected for recovery. The NDELA content was expressed as nanograms/gram urine; the method limit of detection was 0.5 ng/g.

Because the NDELA excretion rate is not constant, 24-hr urine collection does not

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not allow determination of total daily excretion. Concentration, rather than any other parameter, was used to indicate positive NDELA exposure in the workplace.

**Urine mutagenicity detection.** Urine samples were concentrated on XAD-2 columns using the Yamasaki and Ames method (14). The urine adsorbates were tested using the Ames method as modified by Kado et al. (15) at increasing doses, with two strains of *Salmonella typhimurium* (TA98 and TA100), with and without metabolic activation (59% fraction of rat liver induced with Aroclor 1254), with and without β-glucuronidase. All doses were also tested with nicotinamide adenine dinucleotide (NAD) and alcohol dehydrogenase to activate the NDELA to a mutagenic metabolite (9,10).

The urine samples were considered mutagenic according to the modified twofold increase rule (16,17). Therefore, a test was considered positive when two consecutive dose levels produced a response at least twice that of untreated plates and at least two of these consecutive doses showed a dose–response relationship.

**Determination of urine thioethers and D-glucaric acid.** Thioethers were extracted from the urine samples with ethyl acetate, and the thiois were determined spectrophotometrically according to the method of Van Doorn et al. (18). Urinary DGA excretion was determined with the low-pH urinary enzymatic assay of Colombi et al. (19,20). The results of these determinations were expressed as millimoles SH groups or DGA/mole creatinine. We always checked the test performance with standard solutions of N-acetyl-L-cysteine and D-saccharic acid to control variability of the analyses for thioethers and DGA, respectively.

**Blood sampling and SCE determination.** Venous blood was taken from each subject using heparinized vacutainer tubes. Whole blood (0.3 ml) was added within 3 hr of sampling to 4.7 ml RPMI 1640 medium supplemented with 20% fetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin and 5-bromodeoxyuridine (10 µg/ml). Blood was kept at room temperature before the test, since no consistent alteration in SCE frequency was observed for temperatures between 4°C and 37°C up to as long as 48 hr (21). Lymphocyte proliferation was stimulated by 0.1 ml 1 M phytohemagglutinin (22). We incubated the cultures for 72 hr at 37°C (23). We added 0.2 µg/ml of colcemid 69 hr after initiation of cultures (24) and prepared microscope slides using conventional procedures. Staining was performed with the fluorescence plus Giemsa technique (25), and SCE frequency was recorded by observing at least 30 metaphases per sample. Slides were coded and scored blindly by at least two independent observers. The coded slides were scanned under low magnification (100–200X) and selected for scoring on the basis of staining and chromosome number. Only cells with well-differentiated metaphases and 46 elements were accepted for scoring and SCE frequency (26).

**Statistical methods.** The biomarker data were tested for normal distribution using the Shapiro-Wilk test (26), and they were square-root-transformed when appropriate to achieve a better normal approximation or to stabilize variance, as suggested by Lovell et al. (27). The Mann-Whitney rank test was used for comparisons between the two groups, while differences in mean values of SCE, DGA, and thioethers between the high-exposure, low-exposure, and control groups were assessed using analysis of variance (ANOVA). Since age, smoking, and coffee and alcohol drinking can be regarded as possible confounders, models of two-factor analysis of variance were fitted, including categorical variables as factors, age as covariate, and each variable of internal dose or biological effect (biomarker) as response variable (28). More complex models were not used due to the small number of subjects in each group. Validity assumptions of the analysis of variance models were assessed by residual plots (28). The statistical analyses were performed using the 1993 Dynamic version of the BMDP program for personal computer (29). All the statistical tests were two-tailed with an α-value of 0.05; however, p-values between 0.05 and 0.1 were also shown.

**Results**

The distribution of subjects according to exposure, age, smoking habit, and alcohol and coffee drinking is shown in Table 1. The proportion of current smokers was higher among controls (51.4%) than in the high-exposure (32.0%) or low-exposure (18.9%) groups, and a higher proportion of drinkers of ≥23 cups of coffee per day was found in the high-exposure group (48%) than in the others. The mean ages (SD) were 38.0 (9.6), 43.2 (13.0), and 33.4 (9.9) for the high-exposure, low-exposure, and control group, respectively.

The urine samples collected from the high-exposure subjects 4 hr after the beginning of the work shift showed trace amounts of NDELA, from 0.5 to 2.7 ng/g urine; 68% of the subjects had values <0.5 ng/g (below the detection limit), and 32% had values between 0.6 and 2.7 ng/g urine. The urine samples collected from the same subjects before working hours were all NDELA negative. Urine specimens from the low-exposure and the control group were always NDELA negative. Cigarette smoking did not affect NDELA excretion.

According to the qualitative analysis of the mutagenicity test (positive/negative), urine mutagenicity was detectable only

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**Table 1. Categorization of subjects according to occupational exposure to N-nitrosodimethyamine-containing cutting fluids, age, smoking status, coffee consumption, and alcohol drinking**

| Variable                  | High exposure | Low exposure | Total exposed | Controls | Total |
|---------------------------|---------------|--------------|---------------|----------|-------|
| Age (years)               |               |              |               |          |       |
| <30                       | 7 (28.0)      | 7 (18.9)     | 14 (22.6)     | 14 (37.8)| 28 (28.3) |
| 30–39                     | 7 (28.0)      | 11 (29.7)    | 18 (29.0)     | 13 (35.1)| 31 (31.3) |
| 40+                       | 11 (44.0)     | 19 (51.4)    | 30 (48.4)     | 10 (27.0)| 40 (40.4) |
| Smoking status            |               |              |               |          |       |
| Nonsmoker                 | 12 (48.0)     | 25 (67.6)    | 37 (59.7)     | 16 (43.2)| 53 (53.5) |
| Ex-smoker                 | 5 (20.0)      | 5 (13.5)     | 10 (16.1)     | 2 (5.4)  | 12 (12.1) |
| Current smoker            | 8 (32.0)      | 7 (18.9)     | 15 (24.2)     | 19 (51.4)| 34 (34.3) |
| Coffee consumption (cups/day) |            |              |               |          |       |
| 0                         | 3 (12.0)      | 11 (29.7)    | 14 (22.6)     | 4 (16.0) | 18 (18.1) |
| 1–2                      | 10 (40.0)     | 22 (59.5)    | 32 (47.4)     | 12 (48.0)| 44 (44.4) |
| 3+                       | 12 (48.0)     | 4 (10.8)     | 16 (25.8)     | 9 (36.0)| 25 (25.2) |
| Unknown                   | —             | —            | —             | 12 (48.0)| 12 (12.1) |
| Alcohol drinking (g/ethanol/day) |         |              |               |          |       |
| 0                         | 5 (68.0)      | 9 (24.3)     | 14 (22.6)     | 9 (34.6)| 23 (23.2) |
| 1–2                      | 11 (44.0)     | 16 (43.2)    | 27 (43.5)     | 8 (30.8)| 35 (35.3) |
| 3+                       | 9 (36.0)      | 12 (32.4)    | 21 (33.9)     | 9 (34.6)| 30 (30.3) |
| Unknown                   | 1 (2.7)       | 1 (2.7)      | 1 (1.4)       | 11 (28.7)| 12 (12.1) |
| Total                     | 25 (100)      | 37 (100)     | 62 (100)      | 37 (100)| 99 (100)  |

*Percentages in parentheses.

*Low and high occupational exposure: subjects exposed to cutting fluid with NDELA content <5 mg/l and ≥5 mg/l, respectively.
with \textit{S. typhimurium} TA98 strain and S9 mix and only among smokers in the three groups (data not reported). The addition of \textbeta-glucuronidase, NAD, and alcohol dehydrogenase gave negative results.

We compared the mean values of urine thioethers and DGA between the samples taken at the beginning and end of the work shift. In the high-exposure group, a slightly higher mean value was found in the beginning-of-shift samples compared to the end-of-shift samples for both thioethers (from 11.9 to 12.5 SH/creatinine) and DGA (from 3.9 to 4.1 mmol/mol creatinine), although the mean differences were not statistically significant. No differences were found in the low-exposure group (thioethers from 8.6 to 8.7; DGA from 4.1 to 4.9).

A fair correlation was observed between thioether and DGA values measured before and after exposure at an individual level: the Spearman's rank correlation coefficient range was 0.5–0.6 in all groups (\(p<0.001\)). On the other hand, both biomarkers showed a statistically significant interindividual variation by one-way ANOVA in both groups (\(p<0.01\)).

The mean values of urinary thioethers and DGA and lymphocyte SCE according to occupational exposure and smoking habit (current smokers versus nonsmokers and ex-smokers combined) are shown in Table 2. A significant linear increase in the mean thioether values was evident from the control to the lower- and higher-exposure groups (\(p=0.003\)), while controls had a lower DGA mean value compared to the exposed subjects. Smokers had higher values for biomarkers than nonsmokers in all exposure groups. The increase in mean thioether values from the control to the low- and high-exposure groups was more evident among smokers than nonsmokers: in all subjects, the mean values of urinary thioethers increased from 10.8 to 13.1 (21% increase) and 16.1 (50% increase) mmol/mol creatinine in smokers (linear contrast: \(p=0.006\)) and varied from 8.5 to 7.6 (11% decrease) and 10.9 (28% increase) mmol/mol creatinine in nonsmokers (linear contrast: \(p=0.08\)).

Table 3 shows the effect of age, smoking, and coffee and alcohol drinking on biomarkers in all subjects. A linear increase was found in mean values of all the biomarkers according to smoking status and coffee consumption, and a linear increase of lymphocyte SCE was found according to age. These trends were all statistically significant as tested by linear contrasts. The effects of age, tobacco smoking, and coffee drinking were evident in all the subgroups when examined separately (data not shown). In the contrast, alcohol drinking was never associated with any of the biomarkers, therefore it was not considered in further analyses.

At the second stage, the effect of occupational exposure on the biomarkers was assessed, adjusting for age and for smoking status or coffee drinking by two-factor ANOVA with age as a covariate (continuous variable). Coffee drinking was dichotomized, due to the small number of subjects in some categories. Drinkers of \(\geq2\) cups of coffee a day were contrasted with nondrinkers and drinkers of 1 cup/day combined. Three models were fitted, including the following categorical, independent factors (Table 4): occupational exposure to NDELA-containing cutting fluids and smoking status (model A), occupational exposure to NDELA and coffee drinking (model B), and smoking status and coffee drinking (model C). Smoking status was significantly associated with each biomarker in all the models. Occupational exposure was also associated independently with urinary thioethers (\(p<0.05\)) and, to a lesser degree, with urinary DGA (\(p=0.06\)). Coffee drinking showed a strong association with urinary DGA, which was independent of tobacco smoking when both variables were included in the model. In regard to thioethers, the effect of coffee was not uniform across strata, as shown by the significance of the interaction between coffee drinking and occupational exposure: the effect of coffee was evident in the high-exposure group only. The lymphocyte SCE count independently associated with both smoking status and age, but not with occupational exposure.

### Discussion

This study aimed to assess the occupational exposure to cutting fluids containing NDELA through chemical (urine nitrosamine content) and biological analyses (urine mutagenicity, thioethers and D-glucaric acid, and lymphocyte SCE). The effects of age, smoking status, and coffee and alcohol drinking on these biomarkers were also assessed.

The results of assays of urinary NDELA content in subjects exposed to nitrosamine-positive cutting fluids agreed with the results of our previous study (\(15\)). All the workers exposed to cutting fluids containing \(\geq25\) ppm NDELA excreted trace amounts of NDELA only in urine samples collected 4 hr after the beginning of the shift. Workers exposed to cutting fluids containing \(<5\) ppm NDELA showed no detectable urinary NDELA content either before the shift or 4 hr after the shift began. Therefore, the urinary NDELA assay appears to be useful for detecting and monitoring occupational exposure to cutting fluids containing at least 5 ppm NDELA, although the test is strictly time and dose dependent.

Urinary excretion of mutagens was associated with tobacco smoking but not with NDELA exposure. As NDELA is a

### Table 2. Mean values (SD) of urinary thioethers and \(\beta\)-glucaric acid and of lymphocyte SCE according to occupational exposure and smoking status

| Variable       | Biomarkers          | Thioethers (mmol/mol creatinine) | \(\beta\)-Glucaric acid (mmol/mol creatinine) | No. of SCE/creatinine | Metaphase |
|---------------|---------------------|---------------------------------|---------------------------------------------|----------------------|-----------|
| Occupation    |                     |                                 |                                             |                      |           |
| Smoking       |                     |                                 |                                             |                      |           |
| High exposure | Smokers             | 16.1 (2.7)                      | 4.2 (1.0)                                   | 7.0 (0.8)            |           |
|               | Nonsmokers          | 10.9 (4.4)                      | 4.1 (1.4)                                   | 6.1 (1.0)            |           |
|               | Total               | 12.5 (4.6)                      | 4.1 (1.3)                                   | 6.5 (1.2)            |           |
| Low exposure  | Smokers             | 13.1 (5.3)                      | 5.0 (1.8)                                   | 8.0 (1.2)            |           |
|               | Nonsmokers          | 7.6 (3.0)                       | 3.9 (1.3)                                   | 6.3 (1.2)            |           |
|               | Total               | 8.7 (4.1)                       | 4.1 (1.5)                                   | 6.6 (1.3)            |           |
| Controls      | Smokers             | 10.8 (3.4)                      | 5.7 (1.5)                                   | 7.6 (0.7)            |           |
|               | Nonsmokers          | 8.5 (2.7)                       | 4.0 (0.6)                                   | 6.5 (0.6)            |           |
|               | Total               | 9.8 (3.3)                       | 4.9 (1.5)                                   | 7.1 (0.8)            |           |
| \(F\)-test\*  |                     | \(p=0.003\)                     | \(p=0.05\)                                   | NS                   | NS        |

\*One-way analysis of variance for occupational exposure on square-root-transformed data; NS (nonsignificant) = \(p>0.05\).
strong mutagen in *in vitro* tests (9,10), the lack of urinary mutagenicity in the high-exposure subjects may be due either to a relatively low overall exposure to NDELA or to the detoxification of the chemical.

Many potentially genotoxic compounds are detoxified by reacting with endogenous glutathione, giving rise to glutathione conjugates, which are eventually excreted in the urine or the bile as thioethers (30). Determination of the urinary levels of thioethers has been proposed for screening exposure to various genotoxic compounds, especially alkylating agents. Nitrosamines such as NDELA alkylate both DNA and proteins following metabolic activation, then they are detoxified through the glutathione pathway and finally excreted as urinary thioether products (31).

We found that thioether excretion was related to occupational exposure to NDELA and also to cigarette smoking. The increase in thioether excretion due to cigarette smoking has been widely documented (32–35). Likewise, an increase in the mean urinary excretion of thioethers after various occupational exposures to xenobiotics has been reviewed (36). The possibility of false negative results in regard to urinary thioether levels due to occupational exposure has been argued because thioether excretion is only sensitive to relatively high, recent exposures (18,37). In line with these conclusions, we found an increase in the mean level of urinary thioethers only in the high-exposure group 4 hr after the beginning of the work shift. On the other hand, a fair intranidividual correlation was found in samples collected before and after the beginning of the shift, suggesting that some individual factors, especially liver and kidney function, are relevant and should be taken into account. Therefore, we suggest that, in studies of low exposure to NDELA, comparisons of thioether excretion should be made for the same individuals during the work shift, since they are more sensitive than a comparison with unexposed control groups, as is true for other occupational exposures such as styrene (30).

Cigarette smoking showed the strongest association with urinary thioethers, but the effect of occupational exposure to NDELA was still evident when adjusting for both cigarette smoking and age. However, a more noticeable effect of occupational exposure to thioethers was seen in smokers with respect to nonsmokers: the mean values showed a steady, more relevant increase from the controls to the high- and low-exposure groups in smokers than in nonsmokers. The interaction between smoking and occupational exposure was not statistically significant using two-factor ANOVA, although this is most likely due to the small number of subjects and hence the low power of the tests. The finding of a different effect of NDELA exposure in smokers and nonsmokers confirms the results of previous reports. For instance, a recent study among styrene workers showed higher thioether excretion in tobacco smokers than in nonsmokers performing the same tasks (30). This finding suggests that monitoring of urinary thioethers can be more sensitive in detecting exposure when applied to workers who are current smokers.

Also, α-glucaric acid has been proposed for screening xenobiotic exposures because many DNA-damaging agents, including alkylating agents, can be detoxified through glucuronidation as a major route (36, 38,39). However, we did not find any association between NDELA exposure and urinary DGA. This finding was not entirely unexpected because DGA excretion has been found to increase after exposure to some chemicals, such as pesticides, but not to styrene, dioxin, or toluene (36). In a previous study by our group, no increase was found in steel-plant workers exposed to mineral oils with respect to controls (40). A high intranidividual variation in DGA excretion has been described, which may reflect both genetic and environmental factors and may explain these contradictory results (36). We found an association between coffee drinking and DGA urinary values in this study, which suggests that factors other than occupational exposure can influence urinary DGA excretion, and they should therefore be taken into account in occupational and environmental studies. Overall, the urinary excretion of DGA does not seem to be as sensitive as thioether excretion for the screening of occupational exposure to NDELA.

SCE is a well-known biomarker of exposure to mutagens/carcinogens; a large number of chromosome-damaging agents has been shown to induce SCEs (41). Alkylating agents such as nitrosamines have been shown to produce SCEs *in vitro* (41). Many different exposures have been associated with increased SCE frequency, in addition to age. Among them, cigarette smoking, diet, and some occupational exposures, especially exposure to cytotoxic drugs and ethylene oxide, have been often described (41). In line with these observations, we found that current smokers had a higher mean SCE frequency in lymphocytes than ex-smokers and nonsmokers and that the SCE count increased with age. However, the SCE count was not associated with occupational exposure to NDELA, even when taking into account cigarette smoking and age.

In conclusion, occupational exposure to cutting fluids containing NDELA causes urinary excretion of NDELA, demonstrating that this carcinogenic contaminant is adsorbed by humans. Monitoring of nitrite and NDELA content in cutting fluids could be useful in preventing the health effects of occupational exposure to NDELA (13). The increase in thioethers but not in DGA in the urine samples collected during work hours seems to suggest that NDELA compounds are detoxified by reacting with endogenous glutathione more than through glucuronidation, although further research is needed before any firm conclusions can be reached. In contrast,

| Table 4. Two-factor ANOVA of urinary thioethers and α-glucaric acid and of lymphocyte SCE by various models including occupational exposure to N-nitrosodietanolamine-containing cutting fluids, with smoking status and coffee consumption as categorical factors and age as covariate: statistical significance of F-tests |
| Two-factor ANOVA model | Biomarkers |
|-----------------------|-----------|
|                       | Thioethers | α-Glucaric acid | SCE |
| **Model A**           |           |               |     |
| Occupational exposure | p = 0.004 | p = 0.06       | NS  |
| Smoking status        | p < 0.001 | p = 0.006      | p < 0.001 |
| Interaction           | NS        | NS            |     |
| Age (covariate)       | —         | p = 0.02      | p = 0.01 |
| **Model B**           |           |               |     |
| Occupational exposure | p = 0.01  | p = 0.06       | NS  |
| Coffee consumption    | NS        | p < 0.001     | NS  |
| Interaction           | p = 0.02  | p = 0.06      | p = 0.02 |
| Age (covariate)       | —         | p = 0.06      |     |
| **Model C**           |           |               |     |
| Smoking status        | p < 0.001 | p = 0.004     | p = 0.001 |
| Coffee consumption    | NS        | p = 0.003     | NS  |
| Interaction           | NS        | NS            |     |
| Age (covariate)       | —         | p = 0.02      |     |

Occupational exposure according to three categories: controls, low exposure, high exposure. Smoking status: nonsmoker + ex-smoker vs. current smoker; coffee consumption: 0–1 cups/day vs. 2+ cups/day; NS (nonsignificant) = p > 0.05.
urinary mutagenicity assay and lymphocyte SCE frequency do not seem to be relevant for biomonitoring this exposure. Finally, some factors other than tobacco smoking, such as coffee drinking, may influence the mean values of the markers investigated and should therefore be taken into account in any research on occupational exposure to potential carcinogens.

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