Sister Chromatid Exchanges and Micronuclei in Peripheral Lymphocytes of Shoe Factory Workers Exposed to Solvents

Marìa Pitarché,1 Alexander Vaglenov,1,2 María Nosko,2 Sonya Pavlova,4 Vera Petkova,5 Amadeu Creus,1 Hannu Norppà,5 and Ricard Marcos1

1Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Edifici Cn, Universitat Autònoma de Barcelona, Bellaterra, Spain; 2National Centre of Radiobiology and Radiation Protection, Sofia, Bulgaria; 3Department of Hygiene and Medical Ecology, Medical University, Sofia, Bulgaria; 4Clinical Centre of Occupational Diseases, Sofia, Bulgaria; 5Laboratory of Molecular and Cellular Toxicology, Department of Industrial Hygiene and Toxicology, Finnish Institute of Occupational Health, Helsinki, Finland

We examined sister chromatid exchanges (SCEs) and micronuclei (MN; cytokinesis-block method) in cultured peripheral lymphocytes from 52 female workers of two shoe factories and from 36 unexposed age- and sex-matched referents. The factory workers showed an elevated level of urinary hippuric acid, a biomarker of toluene exposure, and workplace air contained high concentrations of various organic solvents such as toluene, gasoline, acetone, and (in one of the plants only) ethylacetate and methylelenediphenyl diisocyanate. The shoe factory workers showed a statistically significant higher frequency of micronucleated binucleate lymphocytes in comparison with the referents. This finding agreed with three preliminary MN determinations (each comprising 27–32 shoe workers and 16–20 controls) performed in one of the plants 2–5 years earlier. The shoe factory workers also had a lower average level of blood hemoglobin than the referents. In contrast, no difference was found between the groups in SCE analysis. Smokers showed significantly higher mean frequencies of SCEs per cell and high frequency cells (HFC) than nonsmokers. Aging was associated with increased MN rates and reduced cell proliferation. Polymorphism of the glutathione S-transferase M1 gene (GSTM1) did not affect the individual level of SCEs; but in smoking shoe workers an effect of the occupational exposure on the frequency of micronucleated cells could be seen only in GSTM1 null subjects. The low prevalence of the glutathione S-transferase T1 (GSTT1) null genotype precluded the evaluation of the influence of GSTT1 polymorphism. Our results show that the shoe factory workers have experienced genotoxic exposure, which is manifest as an increase in the frequency of MN, but not of SCEs, in peripheral lymphocytes. The exposures responsible for the MN induction could not be identified with certainty, but exposure to benzene in gasoline and methylelenediphenyl diisocyanate may explain some of the findings. Key words: glutathione S-transferase M1, glutathione S-transferase T1, micronuclei assays, shoe factory workers, sister chromatid exchange assays, solvent exposure. Environ Health Perspect 110:399–404 (2002). [Online 11 March 2002] http://ehpnet1.niehs.nih.gov/docs/2002/110p399-404/pitarque/abstract.html

During the last few years, genotoxicity biomarkers have received considerable interest as tools for detecting human genotoxic exposure and effects, especially in health surveillance programs dealing with occupational exposure to chemical carcinogens. Currently, only cytogenetic end points in peripheral blood lymphocytes allow a reasonable epidemiologic evaluation of cancer predictivity. The largest databases are available for chromosomal aberrations (CA); high CA level has been associated with increased cancer risk (1,2). Evaluations have also been performed for lymphocyte sister chromatid exchanges (SCEs) and micronuclei (MN), but thus far these biomarkers have not been shown to predict cancer (1,2). These findings may reflect the relatively small databases, the comparatively young cohorts, and variation in techniques, which have made it difficult to standardize individual SCE and MN values obtained from different sources. In a few years, accumulation of more uniform data will make it possible to reassess the value of both SCEs and MN. In the meantime, SCEs and MN are used as biomarkers of genotoxic exposure, as simpler alternatives to CA analysis. In the present study, we applied these methods to monitor solvent-exposed workers from two Bulgarian shoe factories.

People employed in the shoemaking industry are at an increased risk of leukemia and nasal cancer (3), and an excess of mortality due to other types of cancer has also been reported (4–6). Workers in shoe and boot factories are exposed to a mixture of organic solvents, among which toluene and acetone are usually the most common. Neither of these solvents is considered a genotoxin or a carcinogen; the weight of evidence from human in vivo studies suggests that exposure to toluene does not cause somatic cell genotoxic damage (7), although this view has been questioned by recent studies of rotogravure printers (8,9).

The glues and gasoline used in shoe manufacture may contain benzene, which could be responsible for some of the cancers found in shoe workers. Benzene is a well-known clastogen that requires metabolic activation to be mutagenic. The genotoxic metabolites are also thought to play an important role in benzene myelotoxicity and leukemogenesis. The quinone metabolites of benzene can break chromosomes by inducing reactive oxygen species but may also act as aepneuroligens, causing microtubule disruption (10,11). Recent investigations have indicated that structural CA are increased in shoe factory workers exposed to benzene and toluene (12,13).

In a preliminary study of the genotoxic effects of occupational exposure to organic solvents, using the alkaline Comet assay, we did not detect any increase in DNA damage in cryopreserved peripheral blood mononuclear leukocytes from a group of women employed in the two Bulgarian shoe factories (14).

Here we report results of lymphocyte SCE and MN analyses in a larger group of shoe workers from the same plants. We also present MN data from three other cross-sectional studies conducted 2–5 years earlier than the last sampling. In all cases, the concentrations of toluene, gasoline, acetone, ethylacetate, and methylelenediphenyl diisocyanate (MDI) in workplace air and the concentration of hippuric acid (HA) in the urine were determined. To evaluate whether genetically determined individual variations in xenobiotic metabolizing capacity modified individual susceptibility to the possible genotoxic effects of the occupational exposure, we examined the subjects for their glutathione S-transferase T1 (GSTT1) null genotype precluded the evaluation of the influence of GSTT1 polymorphism. Our results show that the shoe factory workers have experienced genotoxic exposure, which is manifest as an increase in the frequency of MN, but not of SCEs, in peripheral lymphocytes. The exposures responsible for the MN induction could not be identified with certainty, but exposure to benzene in gasoline and methylelenediphenyl diisocyanate may explain some of the findings. Key words: glutathione S-transferase M1, glutathione S-transferase T1, micronuclei assays, shoe factory workers, sister chromatid exchange assays, solvent exposure.

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Address correspondence to R. Marcos, Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Edifici Cn, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Telephone: +34-93-581-20-52. Fax: +34-93-581-23-87. E-mail: rmd@cc.uab.es

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Materials and Methods

Subjects studied. We conducted four independent biomonitoring studies of Bulgarian shoe factory workers in 1992, 1993, 1995, and 1997. In the first three years, only workers from plant B (Gabrovo) were examined; the last survey also included subjects from plant A (Sofia). In 1997, we examined 33 workers from plant A and 19 from plant B. The control group consisted of 36 unexposed women from the administrative staff of these plants. The subjects represented about half of the staff in each factory and were chosen randomly. In general, the workers participated in the study only once, except for a few individuals who were included in two or three of the samplings. All subjects gave written informed consent and were interviewed for personal data, duration of employment, and smoking habits. The characteristics of the studied groups are summarized in Table 1. The subjects were all women, except eight control subjects and three exposed workers enrolled in 1992. In all cases, the numbers of smokers among the controls and the exposed subjects were adequately matched, except in the 1993 study, where the number of smokers was higher among the controls than among the exposed. In the shoe factory workers, the total duration of occupational exposure gradually increased during the follow-up period; the difference in the average time of employment in the plant between the first and the last sampling was 3 years.

Exposure measurements. We used special diffusive monitors (Hygitest Co., Sofia, Bulgaria) placed at breath level to obtain workplace vapor samples. The average concentrations of gasoline and methyl-enediphenyl diisocyanate (MDI) were measured by express linear-colorimetric method. We measured environmental levels of toluene, acetone, and ethylacetate with an infrared gas chromatography analyzer Miran 1B2 (Foxboro, MA, USA). We monitored these substances in workplace air according to the Bulgarian analytic protocols (15) and Bulgarian Health Ministry’s Order (16). Because the administrative staff was expected to be exposed to very low environmental levels of organic solvents, compared with the exposed workers, and given the results of earlier studies (17–19), exposure values for control subjects included in this study were taken to be zero. The mean workplace levels (milligrams per cubic meter) of some organic solvents in the shoe factories are shown in Table 2, expressed as time-weighted average (8-hr TWA) values. It is evident that the shoe workers were exposed to a complex mixture of solvents. In most cases, the concentrations of these solvents clearly exceeded the limits recommended by European and American standards (20–22).

As a biomarker of toluene exposure, urine samples were collected in the end of the working day, also from the controls, and were analyzed for HA, according to a method previously described (23). As can be seen from Table 3, the shoe factory workers showed a marked increase in the mean HA levels, with a statistically significant difference from controls [p < 0.001; analysis of variance (ANOVA)] in each sampling. A significant relationship between HA concentrations in urine and toluene air levels was found in the entire population studied (β = 0.651, p < 0.001). The presence of phenol, the main urinary metabolite of benzene, was analyzed (24) in urine samples from 29 shoe factory workers and 16 controls included in the 1992 study. As there were no differences between these groups in urinary phenol levels (exposed: 0.12 ± 0.03 mmol/L; controls: 0.10 ± 0.02 mmol/L; detailed data not shown), the workers did not appear to be exposed to high levels of benzene. However, urinary phenol may not reliably indicate lower benzene exposures from gasoline, but these benzene levels may have genotoxic effects.

We analyzed the glues used in these plants in 1997 for benzene content, using a gas chromatograph with a mass-spectrometric detector (Hewlett-Packard 6890/5972A, Wilmington, DE, USA). We detected no benzene traces in these glues.

Blood collection. Heparinized venous blood samples were drawn from each donor, and hemoglobin values were measured in Bulgaria by standard methods. In the 1992, 1993, and 1995 studies, the blood samples were processed in Bulgaria, and blood cultures were set up in a few hours after sampling. To carry out the 1997 study, the blood samples were sent to Barcelona and were put in culture within 48 hr after the collection. Briefly, the blood samples were centrifuged at 170 g for 5 min, the plasma was removed, and RPMI 1640 culture medium (GIBCO, Eragny, France) was added to each blood pellet to attain its initial volume.

Sister chromatid exchanges. We performed the SCE assay only for blood samples obtained in 1997, following standard methodology (25). Briefly, two whole-blood lymphocyte cultures were set up for each donor, and the cultures were incubated covered from light for 72 hr in the presence of 5-bromodeoxyuridine (BrdU; final concentration 8 µg/mL). The cultures were treated with colcemid for the last 2 hr, and the cells were then harvested, treated with hypotonic solution (0.075 M KCl) for 20 min, and fixed three times with methanol-glacial acetic acid. Microscopic slides were prepared from the fixed cells by air drying, and the slides were stained by the fluorescence-plus-Giemsac procedure (26). We purchased BrdU and Hoechst 33258 from Eastman Kodak.

Table 1. Characteristics of the exposed shoe workers and control subjects studied.

| Year, group | No. | Age, years (mean ± SD) | Duration of employment, years (mean ± SD) | No. of smokers (%) |
|------------|-----|------------------------|------------------------------------------|-------------------|
| 1992       |     |                        |                                          |                   |
| Controls   | 16  | 35.3 ± 7.9             | 9.7 ± 9.5                                | 8 (50.0)          |
| Exposed    | 19  | 38.8 ± 9.4             | —                                        | 12 (63.2)         |
| 1993       |     |                        |                                          |                   |
| Controls   | 27  | 40.2 ± 7.8             | 11.1 ± 10.1                              | 9 (33.3)          |
| Exposed    | 27  | 38.2 ± 8.0             | —                                        | 10 (50.0)         |
| 1995       |     |                        |                                          |                   |
| Controls   | 20  | 38.4 ± 10.3            | —                                        | 12 (60.0)         |
| Exposed    | 27  | 36.4 ± 8.3             | 12.3 ± 9.7                               | 16 (59.3)         |
| 1997       |     |                        |                                          |                   |
| Controls   | 36  | 43.7 ± 8.2             | —                                        | 16 (44.4)         |
| Exposed    | 52  | 40.7 ± 8.8             | 12.8 ± 10.5                              | 29 (55.8)         |

| Year | Plant | Toluene | Gasoline | Acetone | Ethylacetate | Diisocyanate |
|------|-------|---------|----------|---------|--------------|--------------|
| 1992 | B     | 275 ± 123 (100–410) | 746 ± 430 (422–1477) | 628 ± 363 (159–1,003) | —            | —            |
| 1993 | B     | 235 ± 64 (68–418)   | 1,334 ± 247 (450–2,000) | 539 ± 252 (24–1,744) | —            | —            |
| 1995 | B     | 148 ± 104 (18–320)  | 300 ± 128 (150–600)    | 960 ± 855 (55–2,900)  | 93 ± 82 (7–360) | 0.21 ± 0.07 (0.06–0.29) |
| 1997 | A     | 76 ± 11 (66–96)     | 457 ± 45 (392–526)     | 374 ± 17 (341–389)    | 246 ± 105 (164–400) | 0.23 ± 0.08 (0.15–0.34) |

*Measured during an 8-hr workshift, except for diisocyanate, which was measured during a 15-min exposure. Values shown are mean ± SD (range). Νot used.
and we calculated the cytokinesis-block numbers of MN and the frequency of binucleate cells with SCEs per metaphase (25/replicate) on coded slides for each donor, analyzed 50 metaphases with some minor adaptations to the specific conditions of the Sofia and Barcelona laboratories. In both cases, the cultures were set up according to Surrallés et al. (28), with some minor adaptations to the specific conditions of the Sofia and Barcelona laboratories. In both cases, the cultures were incubated at 37°C for 72 hr and, at 4 hr after initiation of the cultures, cytochalasin B (Sigma Chemicals, St. Louis, MO, USA) was added at a concentration of 6 μg/mL. The cells were harvested, treated with a mild hypotonic solution (0.075 M KCl) for 2–3 min, and gently fixed three times using methanol/acetic acid (5:1) solution. Air-dried preparations were made, and the slides were stained in a 10% (v/v) solution of Giemsa in phosphate buffer (pH 6.8) for 20 min.

We examined 1,000 binucleate cells for each subject on coded slides (the same scorer in Barcelona and Bulgaria), and the total numbers of MN and the frequency of binucleate cells with MN (BNMN) were scored. We scored 500 lymphocytes to evaluate the percentage of cells with 1, 2, 3, or 4 nuclei, and we calculated the cytokinesis-block proliferation index (CBPI) according to Surrallés et al. (29).

**Genotype analysis.** We used genomic DNA extracted from leukocytes by standard methods as a template in the GSTM1 and GSTT1 genotype analyses, as described elsewhere (30). Briefly, the GSTM1 and GSTT1 genotypes were determined simultaneously in a multiplex polymerase chain reaction (PCR) approach using β-globin specific primers in addition to the GSTM1 and GSTT1 specific primer pairs. The presence of the β-globin specific signal verified the proper functioning of the PCR reaction, whereas the absence of GSTM1 and GSTT1 specific amplification products revealed the corresponding null genotypes.

**Results**

As shown in Table 3, the hemoglobin values of shoe factory workers were reduced compared to those of nonexposed women. This difference, apparently stemming from the occupational exposure, reached statistical significance (p < 0.001) in the ANOVA.

In 1992–1995, we used the MN assay to assess whether the occupational exposure of the shoe factory workers could exert some genotoxic effects. We observed significant increases in the mean frequency of BNMN of the exposed groups each year in comparison with the respective control groups (Figure 1). Especially noteworthy was the constant increase in the mean frequency of BNMN in the exposed groups from 1992 (38.00, SD 20.18) to 1995 (55.2, SD 17.1), whereas control subjects consistently showed similar baseline values (from 18.6, SD 6.3 to 22.0, SD 10.0).

In the last study, carried out in 1997 and involving both factories, the MN assay confirmed our previous positive findings. Table 4 shows the MN and SCE results, separately for nonsmokers and smokers, among the controls and exposed workers. In the multiple ANOVA, occupational exposure (plant A, plant B, and controls) constituted a statistically significant (p < 0.001) source of variation for the BNMN values. The post hoc least squares difference test showed that workers of plant B had a clearly higher BNMN frequency (p < 0.001) than controls or plant A workers, but also plant A workers differed significantly (p = 0.035) from the controls. We detected no effect on cell-cycle kinetics, measured as CBPI, as a consequence of the solvent exposure.

In the SCE assay (Table 4), we were not able to detect any genotoxic effect related to occupational exposure. We found no correlation between MN and SCEs. The occupational exposure was associated with a slight decrease in PRI (p = 0.048).

Age was a significant covariate (p = 0.006) in the multiple analysis of variance of the 1997 BNMN data, reflecting an...
Discussion

The present study showed an increased frequency of MN in peripheral lymphocytes of shoe factory workers exposed to a mixture of organic solvents. In plant B, where lymphocyte MN frequencies were followed up over 5 years, all four samplings yielded the same result. Although the shoe factory workers did not show any effects in the frequency of SCEs, HFC, or DNA breakage, as measured earlier by the Comet assay (14), our findings indicate the presence of genotoxic exposure at the workplace. This would appear to act via an aneugenic or clastogenic mechanism that does not involve DNA breakage readily detectable by the Comet assay and that does not efficiently lead to SCE formation.

In interpreting these findings, one must consider that although there are several examples of MN induction in human binucleated lymphocytes by known clastogenic exposures in vivo, evidence that the in vivo effects of an aneugen can be expressed in the first in vitro division of a binucleated cell is scanty (31, 32). The binucleated cells that are exclusively analyzed in the cytokinesis-block MN assay have, by definition, completed their first in vitro mitosis, and most MN observed in such cells have been formed in the culture. Thus, an in vivo MN inducer would have to be able to initiate its effect in the resting G0 lymphocyte. Further studies of the shoe factory workers using CA analysis or an MN assay incorporating pancentromeric fluorescence in situ hybridization or anti-kinetochore antibodies to identify the contents of the MN will be useful in identifying the nature of the exposure that produced the excess MN.

Although the glues used in the factories did not contain measurable amounts of benzene, there probably was benzene in the gasoline used as solvent. The air concentrations of gasoline were high, and benzene may well be the principal agent responsible for the observed genotoxic effects. In factory B, the levels of MDI (measured as diisocyanate) were very high and could explain some of the genotoxic effects. MDI is highly reactive and induced CA in human lymphocytes in vitro (33). MN frequencies were clearly higher in plant B than in plant A, and this could be caused by MDI, since diisocyanate was not found at all in plant A. Even in plant B, diisocyanate was not discovered during the first three sample collections. Thus, although it may not be possible to point out a single genotoxic agent at the workplace, benzene in gasoline and MDI may explain some of the findings. A possible indication of the heavy organic solvent exposure of the workers was the consistently lowered blood hemoglobin level, which we also observed in the previous study (14). Anemia and other hematologic abnormalities have been associated with organic solvent exposure in Bulgarian petroleum workers (34).

Several investigations have suggested an increased sensitivity of the GSTM1 null genotype to genotoxicity induced by various exposures, including tobacco smoke (35–47). Such an association was not, however, unequivocally demonstrated in most of the few studies where SCEs or MN were ever, unequivocally demonstrated in most of the few studies where SCEs or MN were used as biomarkers (48–58). Nevertheless, our MN results appear to support the increased sensitivity of the GSTM1 null

Table 4. Mean (± SD) frequencies of BNMN and SCEs in lymphocytes of shoe factory workers of plants A and B and unexposed controls, according to smoking habit (last sampling).

| Group         | No. | BNMN/1,000 cells | CBPI | No. | SCEs/1,000 cells | Percent HFC | PRI |
|---------------|-----|-----------------|------|-----|----------------|-------------|-----|
| Controls      |     |                 |      |     |                |             |     |
| Nonsmokers    | 20  | 24.15 ± 7.49    | 1.89 ± 0.16 | 15  | 7.83 ± 1.04    | 2.67 ± 2.47 | 2.26 ± 0.24 |
| Smokers       | 16  | 22.19 ± 8.45    | 1.87 ± 0.11 | 13  | 9.05 ± 1.73    | 8.37 ± 9.26 | 2.32 ± 0.12 |
| Plant A workers |   |                 |      |     |                |             |     |
| Nonsmokers    | 15  | 31.13 ± 10.01   | 1.93 ± 0.08 | 15  | 7.59 ± 0.86    | 2.53 ± 2.45 | 2.15 ± 0.16 |
| Smokers       | 18  | 25.28 ± 12.13   | 1.89 ± 0.09 | 18  | 8.47 ± 1.10    | 6.11 ± 5.29 | 2.28 ± 0.28 |
| Plant B workers |   |                 |      |     |                |             |     |
| Nonsmokers    | 8   | 47.25 ± 9.10    | 1.85 ± 0.20 | 8   | 7.77 ± 0.80    | 4.25 ± 3.62 | 2.29 ± 0.10 |
| Smokers       | 11  | 43.45 ± 13.47   | 1.95 ± 0.17 | 11  | 8.15 ± 1.54    | 5.17 ± 4.98 | 2.38 ± 0.10 |
| Total         | 36  | 23.28 ± 7.67    | 1.88 ± 0.14 | 28  | 8.40 ± 1.51    | 5.31 ± 7.05 | 2.29 ± 0.19 |
| Plant A workers |   |                 |      |     |                |             |     |
| Nonsmokers    | 33  | 27.94 ± 11.44   | 1.91 ± 0.09 | 33  | 8.07 ± 1.08    | 4.48 ± 4.55 | 2.22 ± 0.24 |
| Smokers       | 19  | 45.05 ± 11.89   | 1.91 ± 0.19 | 19  | 7.99 ± 1.13    | 4.76 ± 4.37 | 2.34 ± 0.11 |

*BNMN/1,000 cells was affected by occupational exposure (p < 0.001) and age (a covariate; p = 0.066) in analysis of variance. Smoking affected no. SCEs/cell (p = 0.006) and HFC (p = 0.004) in analysis of variance.

Table 5. Distribution of GSTM1 and GSTT1 genotypes among exposed shoe factory workers and control subjects in 1997.

| Genotypes      | Controls | Exposed | Total |
|----------------|----------|---------|-------|
|                | No. | Percent | No.   | Percent | No.  | Percent |
| GSTM1 +        | 13  | 36.1    | 19   | 36.5    | 32   | 36.4    |
| GSTM1 +        | 44  | 11.1    | 4    | 3.9     | 6    | 6.8     |
| GSTM1 null     | 4   | 11.1    | 29   | 53.9    | 34   | 50.0    |
| GSTM1 null     | 16  | 44.5    | 26   | 52.9    | 44   | 50.0    |
| GSTT1 null     | 3   | 8.3     | 3    | 5.8     | 6    | 6.8     |

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genotype, because in smokers an occupational exposure effect could be seen only among GSTM1 null subjects. Although this finding is based on a few individuals, it could indicate, for example, that smoking induces GSTM1 activity in GSTM1-proficient individuals, which would protect them from the genotoxic effects of the occupational exposure. Smoking is not known to markedly induce GSTM1 in humans in vivo. At present, the possible effect of GSTM1 genotype on MN formation is not well understood. Our previous studies suggested that the baseline level of MN is decreased in GSTM1 null subjects (55).

In one investigation, the GSTM1 null genotype was described to increase SCEs in smokers, although the difference to smokers, although the difference to

| Group, smoking habit | Genotype | No. | No. BNMM/1,000 cells* | CBPI |
|----------------------|----------|-----|-----------------------|------|
| Controls             |          |     |                       |      |
| Nonsmokers           | GSTM1 null| 7   | 22.43 ± 7.44          | 1.89 ± 0.21 |
|                      | GSTM1 +  | 8   | 22.38 ± 4.81          | 1.89 ± 0.14 |
|                      | GSTM1 +  | 9   | 19.89 ± 6.34          | 1.84 ± 0.15 |
|                      | GSTM1 +  | 5   | 25.20 ± 10.03         | 1.89 ± 0.03 |
| Plant A workers      |          |     |                       |      |
| Nonsmokers           | GSTM1 null| 7   | 30.29 ± 11.24         | 1.91 ± 0.07 |
|                      | GSTM1 +  | 7   | 31.29 ± 10.14         | 1.94 ± 0.08 |
|                      | GSTM1 +  | 11  | 27.82 ± 14.35         | 1.90 ± 0.07 |
|                      | GSTM1 +  | 6   | 21.17 ± 7.19          | 1.91 ± 0.12 |
|                      | GSTM1 +  | 6   | 47.50 ± 11.68         | 1.92 ± 0.13 |
|                      | GSTM1 +  | 3   | 48.33 ± 8.62          | 1.73 ± 0.29 |
|                      | GSTM1 +  | 6   | 45.00 ± 13.83         | 2.00 ± 0.19 |
|                      | GSTM1 +  | 3   | 33.33 ± 12.50         | 1.94 ± 0.20 |

*Post hoc least squares difference test indicated a higher BNMM frequency in the exposed (plants A and B together) than the controls in GSTM1 null subjects among both smokers (p = 0.006) and nonsmokers (p = 0.003), but in GSTM1 positive subjects only among nonsmokers (p = 0.017).

Table 6. Mean (± SD) frequency of BNMM and CBPI in shoe factory workers and unexposed controls, according to smoking and GSTM1 genotype (last sampling).

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