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Genotoxicity study in lymphocytes of workers in wooden furniture industry

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We have evaluated the extent of genetic damage (frequencies of sister chromatid exchange (SCE) and micronuclei) in blood lymphocytes and found a correlation between exposure period and genetic damage, in workers in the wooden furniture industry.

Venous blood samples were drawn from thirty non-smoking workers exposed to wood dust and thirty non-smoking controls. Lymphocytes were cultured for 72 h and at the end of the culture time the cells were harvested, stained and coded for blind scoring. Damage to the genetic material was evaluated by counting the number of SCEs in 100 metaphases and the frequency of micronuclei in 1000 binucleated cells. Mitotic and proliferative rate indices were also determined.

Environmental studies showed a dust level of 0.30 mg/m^3 (particle size 2.5 microns). The mean micronuclei frequency was significantly higher in the exposed group (17.2%; SD = 1.17, $P < 0.001$; range 13–21.1) compared to the controls (7.54%; SD = 1.6, $P < 0.001$; range 5–10.5). The mean value of SCEs was also higher (7.17, SD = 1.80) in the exposed workers than in the controls (6.71, SD = 1.56), but the difference was not statistically significant. A positive correlation existed between the duration of exposure and micronucleus frequency (correlation coefficient, $\gamma = 0.64$) and SCE (correlation coefficient $\gamma = 0.47$). These results indicate that occupational exposure to wood dust causes genetic damage, which may lead to health hazards like cancer, with or without other risk factors.

OCCUPATIONAL exposure to various dust particles is a common cause of respiratory disorders like wheezing and acute and chronic bronchitis in wood-processing factory employees, grain workers and feed-mill employees[1]. Several independent groups have suggested health risk associated with chronic occupational exposure to wood dust[2–7]. These studies reported the occurrence of lower and upper respiratory diseases such as alveolitis allergica, bronchial asthma, aspergillomycosis and rhinitis. The genotoxicity of wood dust can be evaluated using the sister chromatid exchange (SCE) and micronuclei test (MNT). However the frequency of micronuclei serves as the best quantitative measure for structural and numerical chromosome

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aberrations[8]. The other test can normally be used to monitor genetic damage by counting the number of SCEs in cultured human lymphocytes[9].

Extensive studies have been carried out on the allergic effects caused by wood dust of subtropical trees such as Western red cedar (*Thuja plicata*), red wood (*Sequira sempervirens*), obeche (*Triphochiton scleroxylon*) and Cocabolla (*Dalbergia retusa*)[2,3]. It has been shown that sensitization to the dust of hard woods can cause asthma, the symptoms typically occurring during evenings or nights after exposure. The most extensively studied causative agent of occupational asthma is plicatic acid found in the wood dust of Western red cedar wood. The adverse effect of wood dust also depends on its chemical composition and the contents of microorganisms inherent in the dust.

Currently available data are not sufficient to provide a base for correlating wood dust with occupational health hazards. Also, there are no animal models for evaluating the toxicity, mutagenicity and carcinogenicity of wood dust. In most countries where wood and furniture industries flourish, occupational health hazards associated with chronic exposure to wood dust have not been studied adequately. Therefore, the present study was designed to evaluate the extent of genetic damage in terms of frequency of SCEs and presence of micronuclei in the peripheral blood lymphocytes of wood workers, and to find a correlation between exposure duration and genetic damage.

Subjects were chosen from three wooden furniture firms in and around Chennai, engaged in large-scale production of doors and household furnitures using wood of oak (*Quercus suber*), teak (*Tectona grandis*), rosewood (*Dalbergia latifolia*), red sandalwood (*Pterocartus santalinum*), kadamb (*Anthocephalus cadamba*), Vengai (*Pterocarpus marcipium*), shisham (*Dalbergia sisoo*) and vilvam (*Aegla marmelos*). Thirty males exposed to wood dust for a minimum period of two years and thirty non-exposed, non-smoking males were selected with the best possible matching for age. Each subject was aware of the study hypothesis and after written consent, a personal interview was conducted based on a standard questionnaire regarding medication, symptoms, smoking and diseases. The level of air-borne respirable fraction of wood dust to which the workers were exposed was measured using personnel sampler and cyclone holder (Casella, London).

Table 1. Sister chromatid exchange and micronuclei in lymphocyte cultures of workers exposed to wood dust

Age (in years)	SCE (N/cell)	MN (%) (N/1000* 100)	PRI (%)	MI	Exposure duration (in years)
45	8.62	19.3	2.35	6.67	25
50	9.52	21.1	2.4	7.84	25
50	10.72	19.1	1.98	6.48	25
38	9.35	13.0	2.1	6.60	20
32	7.62	18.0	2.0	5.42	18
38	6.73	16.7	2.36	4.82	17
38	7.54	17.6	2.54	5.64	17
33	5.33	18.3	2.31	5.56	15
41	6.32	18.7	2.51	6.76	15
28	5.58	18.0	2.20	6.35	13
38	6.92	17.2	2.00	6.42	13
29	6.24	15.8	2.11	5.56	8
25	5.02	16.3	2.38	7.00	2
28	4.28	17.0	2.42	5.50	12
42	6.93	18.9	1.86	5.28	25
45	6.65	19.0	2.00	6.00	17
30	5.37	17.3	2.18	7.36	14
35	6.53	16.5	2.34	5.48	12
44	7.39	17.5	2.16	7.00	13
35	6.48	15.7	2.63	5.08	7
30	4.55	18.0	2.68	5.20	10
32	5.32	16.0	2.00	5.81	5
46	10.72	18.3	2.17	6.36	14
40	9.59	17.4	1.90	6.40	12
40	8.95	16.5	2.22	6.89	10
39	9.98	17.8	2.24	6.52	11
36	7.63	16.7	2.05	7.48	8
38	7.08	16.6	2.66	7.58	5
27	5.68	13.3	1.80	6.37	3
31	6.34	14.6	2.41	5.29	2
Mean 36.76	7.17	17.2	2.24	6.22	13.1
SD 6.80	1.80	1.71	0.24	0.81	6.67

SCE, Sister chromatid exchange; MN, Micronuclei; PRI, Proliferative rate index; MI, Mitotic index; SD, Standard deviation.

A modified cytokinesis block technique was used to measure micronucleated lymphocytes[8]. Lymphocyte cultures were set up by adding 0.5 ml of whole blood (sodium heparinized) to 5 ml of RPMI-1640 (Himedia Lab Pvt Ltd, Mumbai) medium supplemented with 20% gamma-irradiated foetal bovine serum, antibiotics (penicillin and streptomycin) and 4% of phytohaemagglutinin-M to stimulate the lymphocytes. The cultures were incubated at 37°C and cytochalasin B (3 µg/ml of culture) was added in the forty-fourth hour after the initiation of cultures to block cytokinesis. After 72 h the cells were collected by centrifugation, treated with hypotonic solution (0.01 M KCl), fixed in methanol: glacial acetic acid (3 : 1) and harvested on slides.

The slides were air-dried and stained for 5 min in 4% buffered Giemsa solution. All slides were coded and analysed microscopically at 100 X magnification under oil immersion lens for micronuclei. A total of 1000 binucleated cells were counted and the values were presented as percentage of micronucleus in 1000 binucleated cells.

Half a millilitre (0.5 ml) of whole blood was added to 5 ml of culture medium RPMI-1640 supplemented with 20% gamma-irradiated foetal bovine serum and 4% of phytohaemagglutinin-M to stimulate the lymphocytes. The cultures were incubated at 37°C. After 24 h of initiation of the cultures, 15 µg/ml of 5-bromo-2′-deoxyuridine (Brdu; Sigma Chemical Co, USA) was added. The cultures were incubated at 37°C in the dark. After 71 h colchicine (0.1 µg/ml of culture) was added and an hour later the cells were harvested, given an hypotonic treatment (0.075 M KCl), and fixed in methanol: glacial acetic acid (3 : 1). The slides were dried and stained with giemsa, coded and scored for SCEs. Thirty complete second metaphases were examined to measure the frequency of SCEs. One SCE was counted when two adjacent segments of one of the chromatids in a chromosome were stained differently. The value of SCE for each specimen was taken as the mean rate of the SCEs per metaphase.

The proportion of metaphases in the total cell population was measured to calculate the mitotic index (MI). MI was estimated by counting a total of 1000 cells.

The proliferative index was calculated using the formula:

$$\text{Proliferative index} = (1 \times M_1\% + 2 \times M_2\% + 3 \times M_3\%)/100,$$

where M_1, M_2 and M_3 are the metaphase cells in the first, second and third replication cycle, respectively.

Table 2. Sister chromatid exchange and micronuclei in lymphocyte cultures of the control group

Age (in years)	SCE (N/cell)	MN (%) (N/1000* 100)	PRI (%)	MI
46	8.10	7.8	2.28	6.60
49	8.53	6.0	2.26	7.03
50	9.78	10.0	2.00	5.67
37	9.00	8.5	2.00	6.58
32	6.42	7.3	2.13	5.26
39	6.58	10.5	2.06	4.00
38	7.42	8.4	2.68	5.23
32	5.20	6.2	2.21	5.30
40	6.00	10.2	1.98	6.46
28	5.50	5.2	2.13	6.00
37	6.32	9.6	1.86	6.34
30	6.16	7.8	2.10	5.42
25	5.00	5.0	2.12	6.61
27	4.16	5.0	2.20	5.33
42	5.98	7.6	1.03	4.84
44	6.35	9.7	1.98	5.80
30	5.32	6.9	2.00	7.06
36	6.50	8.0	2.14	4.82
45	7.21	8.5	2.00	6.45
35	5.96	6.0	2.48	4.82
30	4.46	6.3	2.54	5.43
32	4.78	5.8	1.79	5.61
46	8.66	9.3	2.05	6.19
39	8.87	7.8	1.84	6.32
40	8.49	6.7	2.00	6.50
38	9.00	7.8	2.31	6.85
36	7.54	8.2	1.88	7.36
38	7.54	5.6	2.33	6.20
28	4.36	6.2	2.20	6.08
30	6.22	8.4	2.00	5.16
Mean 36.63	6.71	7.54	2.11	5.91
SD 6.75	1.56	1.60	0.22	0.80

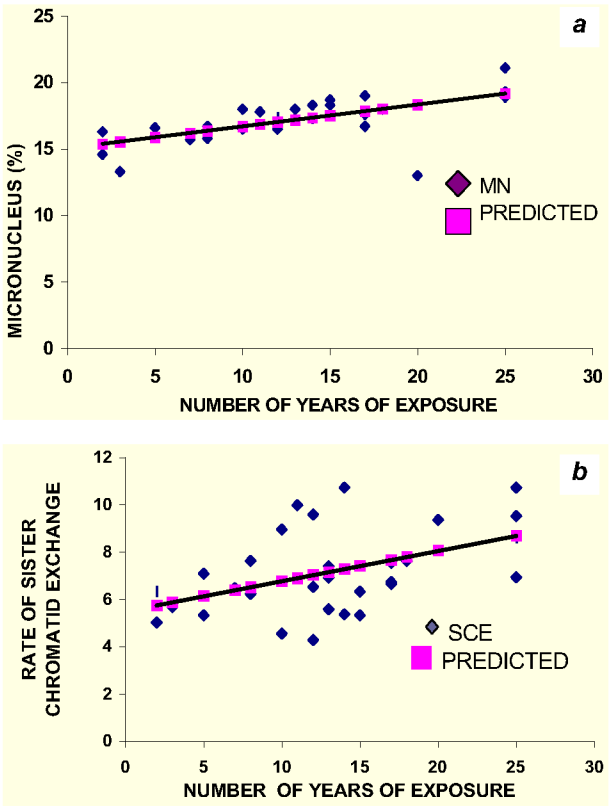

Figure 1. Correlation between (***a***) micronucleus frequency (%) and number of years of exposure; and (***b***) Sister chromatid exchange and number of years of exposure.

Mean ($\bar{X}$) and standard deviation (SD) were calculated for both the exposed and control (non-exposed) groups. An unpaired Student's t test (with 29 degrees of freedom) was applied to determine significant differences of MN frequency between the control and exposed group (α error of 5%). Pearson's correlation analysis was done to find out whether any correlation existed between the number of years of exposure to wood dust, and the MN frequency and rate of SCE. Regression analysis was done to estimate the best fit in both the cases.

The frequency of occurrence of micronucleated lymphocytes was higher in the exposed group (Table 1) than in the control group (Table 2). The value for the exposed group was 17.2% (SD = 1.71; range 13–21.1); for the control group, it was 7.54% (SD = 1.60; range 5–10.5). Significant differences between the values of MN frequency between the exposed and non-exposed groups existed, as shown by the unpaired Student's t test ($P >$ 0.001, 95% confidence interval–8.8 to 10.5).

The rate of SCE was higher in the exposed group (Table 1) than in the control group (Table 2), but the difference was not significant ($P = 0.30$, 95% confidence interval–0.42 to 1.32). The value for exposed group was 7.17 (SD = 1.80) and for control group, it was 6.71 (SD = 1.56).

There was a significant difference in the values for proliferative index between the exposed (Table 1) and control (Table 2) groups ($P = 0.032$, 95% confidence interval–1.2 to 0.25). The mitotic index did not differ significantly ($P = 0.137$, 95% confidence interval–0.10 to 0.72) between the exposed (Table 1) and control (Table 2) groups.

There was a positive correlation between the duration of exposure and MN frequency as shown by Pearson's correlation (correlation coefficient $\gamma = 0.64$) analysis. As the number of years of exposure to wood dust increased, the frequency of micronuclei also increased, indicating increased genetic damage (Figure 1 *a*). Although there was no significant difference in the rate of SCE between the exposed and control groups, correlation analysis showed a positive correlation between the number of years of exposure and rate of SCE (correlation coefficient $\gamma = 0.47$) (Figure 1 *b*).

Several studies indicate that occupational exposure to wood dust can cause upper and lower tract respiratory diseases[9–14]. They could not find a significant correlation between genetic damage and exposed period. The present study confirms that workers exposed to wood dust show genotoxic effects, and the level of genetic damage increases with the increasing years of exposure. In contrast to other studies, we have documented that the respirable level of wood dust ranged between 0.27 and 0.30 mg/m^3. As in the study of Liou *et al.*[15], we found that the total dust concentration ranged from 4.5 to 27.3 mg/m^3. The measured values were at higher levels. It is therefore recommended that the risk should be minimized by reducing exposure to the greatest extent possible.

Formation of micronuclei is one type of chromosomal aberration reflecting genomic instability, whether inherent or acquired[16]. It is assumed that their presence is due to the loss of a whole chromosome or a fragment of a chromosome from the nucleus. Although the micronucleus test is widely used to study genotoxic potential, there is but one report about the formation of micronuclei in workers exposed to birch-wood dust[17]. Unfortunately, this study reporting air concentration of wood dust and micronuclei frequency among 83 workers, did not present data on exposure duration, work-place conditions and correlation. In our study, the frequency of micronuclei was significantly higher in the exposed group than in the controls and there was also a positive correlation between the number of years of exposure and frequency of micronuclei. These effects thus reveal that chemical contents of wood dust manifest toxicity by affecting nuclear DNA function through various mechanisms.

The SCE test is widely used to screen chemicals for their possible genotoxic character. No previous reports on occupational exposure to wood dust and SCE have been published. We found that the rate of SCE among workers exposed to wood dust was higher than that of controls. The results, however, were not statistically significant. A positive correlation existed between the number of years of exposure and the rate of SCEs. The increased rate of SCE and frequency of micronuclei in wood workers may partly explain the increased risk of cancer or other health hazards to the lung and other sites. As with previous studies relating to similar working conditions, we have also documented various symptoms and diseases of the respiratory system that prevailed among the subjects, viz. wheezing, continuous cough, asthma, bronchitis, rhinitis and cancer (reported in two cases). Similarly, Maciejewska *et al.*[18] have documented bronchial asthma, alveolitis allergica and rhinitis among wood workers. In spite of these results, our study had a limitation, viz. we could not distinguish between the potential genotoxic effects of individual wood dust since all the wood was processed in the same place, sometimes as a mixed lot. Further, studies are needed to gain knowledge on the events associated with different kinds of wood dust and genetic damage.

It can be concluded from this study that chronic occupational exposure to wood dust leads to an increased risk of genetic damage among workers of wooden-furniture industries. Further, the genetic damage found may lead to health hazards like cancer, with or without other risk factors. In-depth studies are yet to be initiated to gain knowledge on the primary level of damage that could possibly occur in the lungs and the respiratory tract. Also, safety measures have to be improved to prevent occupational health hazard in the wooden-furniture industry.

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Binary cloning vectors for efficient genetic transformation of rice

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The availability of effective vector systems is a prerequisite for genetic manipulation of plants through recombinant DNA technology. We report here construction of a series of binary vectors that have cauliflower mosaic virus 35S promoter-driven genes encoding either resistance to hygromycin or phosphinothricin for selection of the transformants, and high strength constitutive promoters of either *ubiquitin1* or *actin1* genes for efficient expression of the transgenes. The efficacy of the constructs is tested in stably transformed Pusa Basmati 1 rice plants through β-*glucuronidase* reporter gene activity. Availability of vectors with variable promoters and selectable marker genes provides flexibility in stacking two genes. The vectors constructed in this study are suitable for both particle gun and *Agrobacterium*-based transformation protocols.

RICE is the most important food crop. Of late, rice has emerged as a model crop for the analysis of genome and proteome of cereals[1,2]. There is a great deal of progress in stable genetic transformation of rice plants. Compared to conventional breeding, genetic engineering is a relatively faster means for varietal improvement that allows transfer of genes from within as well as outside the primary gene pool[3]. Genetic transformation of rice through use of *Agrobacterium* is a favoured approach as it enables transfer of DNA with defined ends, minimal rearrangement, integration of a small number of copies of the gene and more importantly, the possibility that even large segments of DNA can be efficiently transferred[4–8].

Success achieved in genetic improvement of most of the agronomic characteristics in rice (as well as in other cereals and dicot plants) is limited. Despite the massive support given to rice biotechnology programme in recent years, there is not much success in improving resistance against biotic and abiotic stresses that cause significant damage to rice yield[3,9]. An important reason for this failure is that mostly the stress resistance-related traits are mediated by a number of biochemical reactions/physiological processes, and methods for co-integration of multiple transgenes into the plant genome are not well established.

Earlier studies have shown that *Agrobacterium*-mediated gene pyramiding can possibly be brought about by either sequential transformation or co-transformation with multiple T-DNAs[10–12]. The multiple T-DNAs can be delivered to plant cells either from a mixture of strains (mixture method) or from a single strain which contains more than one T-DNA (single-strain method)[10]. However, the important parameters that must be noted while designing a co-transformation experiment are that (a) there should be different promoters available to drive different transgenes in the same host, so as to avoid homology-based recombination and gene silencing[13], and (b) there should be different selectable markers available to select each transgene separately. We have used two different promoters obtained from *ubiquitin1* and *actin1*

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