Effect of NO\(_x\) on the Somatic Chromosomes of Goldsmiths

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The genotoxic effect of NO\(_x\) was investigated on somatic human chromosomes obtained from lymphocytes of 45 goldsmiths exposed to 1770.5 mg/m\(^3\) NO\(_x\) in ambient air at normal temperature and pressure and compared to an equal number of matched controls breathing air containing 50 \(\mu\)g/m\(^3\) NO\(_x\). Short-term lymphocyte cultures were set up from blood collected from both exposed and control individuals by venipuncture in heparinized sterile syringes. Mitotic index (MI), chromosome aberrations (CAs), sister chromatid exchanges (SCEs), and satellite associations (SAs) were analyzed. All the parameters showed a significant increase (\(p<0.01\) and \(p<0.05\)) in the exposed individuals as compared to the controls: MI (9.57 vs. 5.01), CAs (3.48 vs. 0.711), SCEs (10.56 vs. 7.02), and SAs (25.97 vs. 12.84), respectively. Occurrence of DG-type SAs (one D-group chromosome and one G-group chromosome) was highest and 3 D-type (three D-group chromosomes) lowest. NO\(_x\) was thus found to be genotoxic for humans. Key words: chromosome aberrations, genetic hazards, genotoxicity, mitotic index, NO\(_x\), satellite association, sister chromatid exchange. Environ Health Perspect 106:643–647 (1998). [Online 4 September 1998] http://ehpnet1.niehs.nih.gov/docs/1998/106p643-647yadav/abstract.html

NO\(_x\) (a mixture of NO2 and NO gas) is a red brown toxic gas. When inhaled through the respiratory tract and absorbed through the skin, it causes various skin diseases and respiratory problems. Based on in vitro studies, Beckett et al. (1) postulated that the environmental concentration of HNO\(_x\) is formed within the respiratory system predominantly by hydrogen abstraction, with subsequent conversion of HNO\(_x\) at physiologic pH, to \(\text{H}^+\) and NO\(_x\). Victorin (2) proposed that HNO\(_x\) formed in this way may contribute to the bronchoconstricting effects of NO\(_x\) seen in normal subjects and asthmatics. Victorin (2) also observed eye irritation just before, during, and immediately after exposure of asthmatic patients to NO\(_x\). Eye irritation was reported to be significantly higher in individuals with HNO\(_x\) exposure than in controls. Moreover NO\(_x\) levels as low as 0.5 ppm increase susceptibility to bacterial infection of lungs (2).

In India, laborers are rarely aware of the potential danger that can accumulate in their genomes due to pollutants in workplaces. NO\(_x\) gas, evolved from aqua regia used by goldsmiths, is such an example. When gold or silver is added to aqua regia (a mixture of HNO\(_x\) and HCl), copper and other metal impurities present in these metals are reduced with the evolution of brown fumes of NO\(_x\) gas. This prompted us to screen goldsmiths for genetic damage.

Materials and Methods

This study included 45 goldsmiths exposed to NO\(_x\) and 45 controls matched with respect to age, sex, smoking habits, drinking habits, and social status. Through spectrophotometry the average concentration of NO\(_x\) in the workplace was found to be 1770.05 mg/m\(^3\) compared to 50 \(\mu\)g/m\(^3\) at normal temperature and pressure.

We conducted an epidemiological survey using a proforma especially designed for this purpose. After obtaining consent, we performed subsequent medical examinations of subjects suffering from asthma.

Short-term lymphocyte cultures were prepared from heparinized blood (heparin 500 IU/ml without preservative) according to the method of Moorehead et al. (3), with slight modifications. Blood (0.5 ml) was added to 5 ml RPMI 1640 medium containing 20% fetal calf serum, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 0.1 ml phytohemagglutinin (Sigma, St. Louis, MO). Colchicine (10 \(\mu\)g/ml) was added to the culture 2 hr before harvesting.

Lymphocytes were harvested after 48 hr for determination of mitotic index (MI), chromosomal aberrations (CAs), and satellite associations (SAs). Slides were air dried and stained with 4% Giemsa. As many as 100 good metaphases (well-spread metaphases showing 2N-46 and proper morphology of chromosomes) per individual were screened for CAs.

To determine sister chromatid exchanges (SCEs), we added 5-bromodeoxyuridine (10 \(\mu\)g/ml/culture; Sigma) 24 hr after preparing cultures. We used the method of Perry and Wolff (4) with the following modifications: 1) the cells on slides were stained directly in excess solution of Hoechst 33258 (50 \(\mu\)g/ml in H\(_2\)O; Sigma); 2) the slides were exposed to long-wave UV light of about 320–400 mm intensity; 3) the slides were rinsed with water and incubated for 15–30 min in 2X SSC at 65\(^\circ\)C; and 4) the slides were rinsed with water and stained with 2–3% Giemsa in phosphate buffer (pH 6.8). For calculating the frequency of SCEs per cell, 25 metaphases per individual were analyzed as per international practice.

For calculation of MI, 5,000 cells per individual were scanned from Giemsa-stained slides. The mitotic index was calculated using the formula

\[
\text{MI} = \frac{\text{No. of dividing cells}}{\text{Total no. of cells scored}} \times 100
\]

For evaluating the frequency of SAs, 100 good second division metaphases per individual were scanned. The following criteria given by Hansson (5) were applied: satellite ends of the associating chromosomes had to be directed towards each other with their longitudinal axes meeting between their short arms, and the distance between the centromeres of associated chromosomes could not exceed the total length of one G chromosome, its satellite excluded.

The samples were analyzed blindly by two observers to remove possible laboratory scoring bias. For statistical analysis of results, the Student’s t-test was applied.

Results

The epidemiological survey showed that 6 individuals out of the sample of 45 goldsmiths were asthmatic. As many as 30 persons also complained of irritation in the eyes.

The data obtained during this study are presented in Tables 1–8. It is evident from Tables 1 and 2 that mean MI in exposed workers (9.57) was significantly higher (\(p<0.01\)) than in matched controls (5.01). It was highest in workers with an exposure period of 0–5 years. Thereafter, the MI showed a gradual decline.

We found chromosomal aberrations to be elevated in the group exposed to NO\(_x\). Yields of aberrations in goldsmiths are presented in Table 3, and raw data are given in Table 4. Frequencies of all types of CAs such as dicentrics, rings,acentric fragments, chromatid gaps, breaks, and isochromatid aberrations were significantly higher in the...
exposed sample than in controls. The frequency of total CAs per 100 metaphases was 3.48 in goldsmiths and 0.711 in controls; the difference was significant ($p < 0.01$).

Frequency of SCEs (Table 5) in goldsmiths was significantly higher (10.56; $p < 0.01$) than in controls (7.02). Both among exposed workers and controls, individuals who drink alcohol showed higher SCE frequency (10.30) than controls (7.31). Similarly exposed tobacco smokers showed a higher frequency of SCEs (10.31) than control smokers (7.37). The highest frequency of SCEs (11.02) was observed in an exposed worker who was both a drinker and a smoker (see Table 6). SCEs showed an elevation in frequencies with increase in the duration of exposure (Table 7).

Frequencies of SAs in both exposed and control groups are shown in Table 8. The exposed group showed more than a twofold increase in the frequency of SAs (25.97) compared to controls (12.84). DG association (one D-group chromosome and one G-group chromosome) was found to be highest (7.428%), while 3D association (three D-group chromosomes) showed the lowest frequency (1%).

**Discussion**

In the present study, 30 out of 45 goldsmiths complained of eye irritation. These 30 persons include 6 asthmatic workers. We concluded that fumes of NO$_x$ cause eye irritation in goldsmiths, and the effect is more pronounced in asthmatic goldsmiths. However, genotoxicity per se has no direct relationship either with asthma or eye irritation. These abnormalities only depict general deterioration in health due to NO$_x$ exposure.

There was a significant increase in total CAs in the exposed group as compared to the controls. Chromatid gaps were not taken into account because their significance in cytological monitoring of populations is still a matter of discussion (6). The total chromatid aberrations were more than the total chromosome aberrations in both goldsmiths and controls. A similar elevation in CAs was reported earlier in human populations exposed to polycyclic aromatic hydrocarbons.
Table 5. Frequency of sister chromatid exchanges (SCEs) (25 cells per individual) in goldsmiths and controls as shown by alcohol drinking and tobacco smoking status

|                          | Exposed | Control |
|--------------------------|---------|---------|
| Parameters               | n       | Mean ± SE |
| Total SCEs               | 45      | 10.56 ± 0.09 |
| Drinker                  | 2       | 10.30 ± 0.45 |
| Drinker/smoker           | 1       | 11.02 ± 0.56 |
| Smoker                   | 6       | 10.31 ± 0.28 |
| Nondrinker               | 36      | 10.29 ± 0.10 |
| Nonsmoker                | 36      | 5.69 ± 0.08 |

SE, standard error.

*Value = 13.1; significant at p<0.01.

(PAHs), nitrosoamines, carbon monoxide, and sulfur dioxide (7). A significant elevation in the frequency of CAs in peripheral blood lymphocytes among persons belonging to a given group may indicate an increased cancer risk (8,9). Au et al. (10) and Santos-Mello et al. (11) reported that CAs are initial events in carcinogenesis and constitute an early warning signal for development of cancer. Lu et al. (12) treated mice and rats intragastrically with nitrite in doses of 1.7-47 mg/kg body weight and rabbits were given the same dose in drinking water for 3 months; CAs in bone marrow were induced in all three species.

In this study, we also found a high frequency of dicentrics. Dicentrics are typical and are normally used as a dosimeter for ionizing irradiation. The present situation, however, reveals that nitric oxides are one of the few NOs that may confound biological radiation dosimetry. Moreover, dicentrics are known to be lethal to cell proliferation, implying that carcinogenic risk, as discussed by Awa (9), originates mainly from CAs that survive cell division, the so-called stable aberrations.

The frequency of SCEs in goldsmiths was higher than in controls. Both among exposed workers and controls, alcohol drinkers and tobacco smokers showed higher frequencies. A 1-8 ppm concentration of NO2 in culture bottles for a duration of 2 hr induced SCEs (13). Elevation in the level of SCEs in the human population exposed to PAHs, nitrosamines, carbon monoxide, and sulfur dioxide as compared to controls has also been reported (7). The elevated SCE level in operating room personnel exposed to waste anesthetic gases (mostly halothane, nitrous oxide, and isoflurane) indicates that these gases could be possible genotoxic hazards (14). In human populations exposed to nitrate concentrations in the range of 50-300 mg/l, no increase in SCE frequency was observed (15). However, CAs (12,16-19), SCEs (18), and gene mutations (16,20) were induced in cell cultures after treatment with high doses of sodium nitrite.

Table 6. Raw data showing frequency of sister chromatid exchanges (SCEs) in goldsmiths and controls as shown by alcohol drinking and tobacco smoking status

| Case no. | Duration of exposure (years) | Dri/Sm status | SCE |
|----------|-------------------------------|---------------|-----|
| 1        | 6-10                          | Dri           | 10.65 |
| 2        | 11-15                         |               | 9.95 |
| 3        | 0-5                           | Sm            | 9.68 |
| 4        | 11-15                         | Sm            | 10.99 |
| 5        | 0-5                           | Sm            | 9.86 |
| 6        | 11-15                         | Sm            | 10.39 |
| 7        | 0-5                           | Sm            | 10.58 |
| 8        | 11-15                         | Sm            | 10.38 |
| 9        | 11-15                         |               | 11.02 |
| 10       | 16-20                         |               | 10.98 |
| 11       | 16-20                         |               | 10.86 |
| 12       | 16-20                         |               | 10.57 |
| 13       | 16-20                         |               | 11.06 |
| 14       | 16-20                         |               | 11.68 |
| 15       | 16-20                         |               | 11.62 |
| 16       | 16-20                         |               | 10.23 |
| 17       | 11-15                         |               | 11.25 |
| 18       | 11-15                         |               | 11.06 |
| 19       | 11-15                         |               | 11.14 |
| 20       | 11-15                         |               | 11.56 |
| 21       | 11-15                         |               | 11.76 |
| 22       | 11-15                         |               | 10.46 |
| 23       | 11-15                         |               | 11.94 |
| 24       | 11-15                         |               | 11.38 |
| 25       | 0-5                           |               | 8.08 |
| 26       | 0-5                           |               | 8.32 |
| 27       | 6-10                          |               | 10.60 |
| 28       | 6-10                          |               | 10.59 |
| 29       | 6-10                          |               | 10.58 |
| 30       | 6-10                          |               | 10.50 |
| 31       | 6-10                          |               | 10.00 |
| 32       | 6-10                          |               | 10.61 |
| 33       | 6-10                          |               | 10.51 |
| 34       | 6-10                          | Dri/Sm        | 11.02 |
| 35       | 6-10                          |               | 10.50 |
| 36       | 6-10                          |               | 10.60 |
| 37       | 6-10                          |               | 10.69 |
| 38       | 6-10                          |               | 10.80 |
| 39       | 6-10                          |               | 10.80 |
| 40       | 6-10                          | Sm            | 10.69 |
| 41       | 6-10                          |               | 10.58 |
| 42       | 6-10                          |               | 10.80 |
| 43       | 6-10                          |               | 10.25 |
| 44       | 6-10                          | Dri           | 9.95 |
| 45       | 6-10                          |               | 8.11 |

Abbreviations: Dri, drinker; Sm, smoker; --, nondrinker/nonsmoker. Cases 1-45 are individuals exposed to NO2, and cases 46-90 are controls.

Beyond 5 years, the MI showed a slight decline. Similar declines in MI were reported in workers exposed to SO2 over 5 years (21) and in workers exposed to NH3 over 10 years (22). Rupa et al. (23) observed a decrease in MI in smokers exposed to pesticide for longer periods. The hypothesis that the increased amount of pollutants in exposed individuals could destroy the lymphocytes, resulting in decline of MI (22),

Table 7. Frequency of sister chromatid exchanges (SCEs) in goldsmiths with exposure to NO2

| Duration of exposure (years) | No. of individuals | SCEs ± SE |
|------------------------------|--------------------|----------|
| 0-5                          | 6                  | 9.00 ± 0.24 |
| 6-10                         | 18                 | 10.57 ± 0.15 |
| 11-15                        | 14                 | 10.99 ± 0.17 |
| 16-20                        | 7                  | 11.00 ± 0.25 |

SE, standard error.

Table 8. Frequency of satellite association pattern observed in goldsmiths and controls

| No. of Individuals | Total cells scanned | Type of satellite association | Associations per cell ± SE |
|--------------------|---------------------|------------------------------|---------------------------|
| Exposed (45)       | 4,500               | D-D                           | 225 ± 0.71                |
| Control (45)       | 4,500               | D-G                           | 405 ± 0.71                |
|                    |                     | G-G                           | 180 ± 0.71                |
|                    |                     | 2D-G                          | 135 ± 0.71                |
|                    |                     | 2G-D                          | 90 ± 0.71                 |
|                    |                     | 2G-2D                         | 89 ± 0.71                 |
|                    |                     | 3D                            | 94 ± 0.71                 |
|                    |                     | Total                         | 1,169 ± 0.71              |
|                    |                     | 3G                            | 25.97 ± 0.71              |
|                    |                     | 3D                            | 12.8 ± 0.4                |

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may be applicable in the present case, albeit at a shorter exposure period. A decline in MI with increasing dose of extract of particulate matter has also been reported (24).

Human cancers are reflections of sustained cell proliferative factors (consisting of chemical agents, hormones, etc.) because nondividing cells in adults, such as nerve cells and cardiomyocytes, never develop tumors (25). It has also been reported that some PAHs, which are genotoxic carcinogens, have the capability to increase cell proliferation (26,27). Earlier, Cohen and Ellwein (28) suggested that potential to cause proliferation is common to carcinogens, regardless of whether they have genotoxic potency.

As such, an increased proliferative/mitotic index does indicate genotoxicity/carcinogenicity of the chemical to which the cells were exposed.

We observed a twofold increase in the frequency of SAs per cell. The difference is significant and may lead to increased probability of chromosomal translocations. The frequency of DG-type associations was highest, whereas the frequency of 3D-type associations was lowest. Similar results were reported earlier for workers exposed to SO2 (21) and NH3 (22) and for grape garden workers occupationally exposed to different pesticides (29). Trezepiruz et al. (30) found that chromosomes 21 and 22, which are smaller than chromosomes 13, 14, and 15, possess more extended nucleolus organizer regions; consequently, acrocentric chromosomes 21 and 22 enter into associations more frequently than chromosomes 13, 14, and 15.

A high incidence of SAs has often been considered as a predisposition to an increased tendency of nondisjunction in satellite chromosomes and thus to the induction of D and G trisomies (31). Klossoglu et al. (31) reported cases of increased SAs in families in which such trisomies occur. Among four families in which new cases of translocation trisomies were observed, the trisomies and their parents had an unusually high incidence of SAs (32).

Hansson (5) suggested that the tendency of SA is genetically controlled and specific environments may influence the SA. In the present study, NO2 in ambient air increased SA in exposed goldsmiths to twice that of controls. The controls were exposed to a background frequency of 0.266 ppb NO2, which is near international standards: World Health Organization, 0.25 ppb (33) and EPA, 0.23 ppb (34).

Nitrous acid (HNO2) deaminates bases in DNA; this is one mechanism for direct mutagenic action. Alkylating agents are formed by nitrosation of primary amines. The formation of N-nitroso compounds from secondary amines and amides is another way for indirect mutagenic activity (35).

Nitrite and nitrate are found in blood after inhalation of NO (36), and nitrosated compounds are formed after inhalation of NO2 (37). Nitrosating agents are formed in the skin when mice are exposed to 50 ppm NO2 for 4 hr; most of these nitrosating agents were derived from cholesterol and some from triglycerides via a peroxidized product (38).

Nitrite derived from nitrate may react in vivo with amines and amides to form N-nitroso compounds, which may have carcinogenic properties. Analysis of urine samples of subjects exposed to nitrates in drinking water revealed the presence of N-nitroso compounds (39). NO2 is also known for its potential to produce free radicals and other potent oxidizing species that may oxidize tissue unsaturated fatty acids. Tabacova (40) suspected that peroxidation of membrane lipid is a primary mechanism for the toxicity of NO2-induced lipid peroxidation in maternal tissues and is related to its embryonic effect.

NO may react intracellularly, or in the medium, with any dissolved oxygen or oxygen-derived radicals that may be generated during exposure to NO2, resulting in the formation of NO4 (41). The combination of NO with NO2 yields a potent nitrosating agent, which has been shown to react with secondary amines to yield carcinogenic N-nitrosamines both in aqueous and lipid phase (42). Two mechanisms for this reaction have been postulated involving either nucleophilic attack by an amine on N2O4 or a two-step process of radical reaction with the amine (43). While secondary N-nitrosamines generally require metabolic activation for mutagenicity (44), nitrosation of primary amines, as found on DNA bases, results in immediate deamination and DNA codon alteration (45). This latter mechanism may, at least in part, be responsible for the observed mutagenic effects of NO in our system.

Deamination of cytosine leads to the formation of uracil, which, if not repaired, causes a base substitution mutation. Likewise, the deamination of methylcytosine results in the formation of a thymine residue in the DNA chain, which is repairable. NO has recently been shown to alter bases in both nucleosides and DNA under aerobic conditions and at physiological pH (46,47). Accordingly, exposure to NO could result in the expected deamination of cytosine to uracil and account for the observed mutagenicity of NO.

These results have significant implications for evaluating the genotoxic potential of NOx, especially that of NO, because in most situations involving exposure to high concentrations of NOx, nitric oxide is present in greater concentration relative to NO2. The ability of NO to traverse the cell and sequester in the lipophilic environment within the DNA chain may be crucial to its mutagenic activity (41).

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