MITIGATION OF GENOTOXIC EFFECTS OF FLUORIDE AND ARSENIC BY ASCORBIC ACID IN HUMAN LYMPHOCYTE CULTURES

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SUMMARY: The in vitro effects of arsenic and fluoride added, individually and in combination, to human lymphocytes on the cell cycle proliferative index (CCPI)/replicative index (RI) and sister chromatid exchange (SCE) using fluorescence plus Giemsa staining were examined. A significant increase occurred in SCE per metaphase (SCE/chromosome and SCE/cell) and inhibition of proliferative kinetics, which resulted in a decline of the replicative index in comparison to the controls. The treated lymphocytes also exhibited a reduction in % M1 and % M2 plates but an increase in % M3 plates. The beneficial effects of adding ascorbic acid were clearly evident with recovery in the replicative index. Genotoxic effects induced by fluoride and by arsenic were therefore reversed by ascorbic acid.

Key words: Arsenic and genotoxicity; Ascorbic acid; Cell cycle proliferation index (CCPI); Fluoride and genotoxicity; Genotoxicity in vitro; Sister chromatid exchange (SCE).

INTRODUCTION

There are conflicting reports in the literature regarding genotoxic effects of fluoride. Jachimczak and Skotarczak found that sodium fluoride induces chromosome aberrations in cultured human leucocytes, whereas others observed no significant changes in SCE in bone marrow cells after ingestion of fluoride by mice and rats. Thompson et al. also found that fluoride did not increase the frequencies of chromosomal aberrations or sister chromatid exchanges (SCEs) in human lymphocyte cultures. However, Sheth et al. observed an increase in the frequency of SCEs in endemic human population of North Gujarat, India, as compared to control population. Joseph and Gadhia also demonstrated that the rates of SCEs and chromosome aberrations in persons in an endemic village with high fluoride water in South Gujarat, India, were significantly higher than in another village with low fluoride.

Investigations of genotoxic effects of ingested arsenic have yielded mixed results. In humans exposed to Fowler’s solution (1% KAsO2 in H2O), increased SCEs but no increase in chromosomal aberrations was found, while another study reported increased chromosomal aberrations. Clearly, the foregoing information indicates there is controversy regarding the genotoxic effects of fluoride and arsenic, and further investigations are needed to clarify this important issue.

There are therapeutic agents capable of minimizing the genotoxicity of various natural and man-made mutagens in our daily life. These agents, which have gained increasing importance, include vitamins, sulphydryl substances, and plant products. Here the protective effects of ascorbic acid (vitamin C) on geno-
toxicity of fluoride and arsenic treated cultures were investigated since there are reports suggesting it has ameliorative effects on other sources of chemical toxicity in lymphocyte cultures.\textsuperscript{13,14}

**MATERIALS AND METHODS**

*Subjects:* Venous blood from ten normal, healthy, non-smoking, adult Indian volunteers, 20–25 years old (5 males and 5 females), was collected after obtaining their consent, in sterile heparinized syringes at our departmental clinical facility.

*Peripheral blood lymphocyte culture (PBLC):* After collection of peripheral blood, microcultures were set up in duplicate for each individual according to the standard protocol of Hungerford.\textsuperscript{15} Seven milliliters of RPMI 1640 culture medium (Hi-media, Mumbai, India, pH 7.4) pre-supplemented with 10% fetal calf serum (Centron Laboratories, Mumbai, India) and antibiotics (benzyl penicillin and streptomycin) were dispensed into dark culture vials along with four drops of heparin (Biological E. Ltd, Hyderabad, India) and 100 µL of phytohemagglutinin (5 mg/5 mL distilled water) (Sigma-Aldrich Chemicals, St Louis, MO, USA). After gently shaking the blood samples, 0.5 mL of the whole blood and the following test chemicals (µg/7 mL culture media as listed in Table 1) were added during the time of culture setting: sodium fluoride (Loba Chemie, Mumbai, 99% purity), arsenic trioxide (Maniyar Chemie, Mumbai, 99% purity), and ascorbic acid (Loba Chemie, Mumbai, 99% purity).

![Table 1. Experimental protocol](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (µg/7 mL of culture media)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control, Untreated (Control + distilled water) (Con)</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Positive control (Control + ascorbic acid) (AA)</td>
<td>12</td>
</tr>
<tr>
<td>III</td>
<td>Sodium fluoride (NaF)</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>Arsenic trioxide (As$_2$O$_3$)</td>
<td>0.001</td>
</tr>
<tr>
<td>V</td>
<td>NaF + As$_2$O$_3$</td>
<td>10 + 0.001</td>
</tr>
<tr>
<td>VI</td>
<td>NaF + As$_2$O$_3$ + AA</td>
<td>10 + 0.001 + 12</td>
</tr>
</tbody>
</table>

All the test compounds were added at ‘0’ hour i.e. during the time of culture setting.
Number of volunteers = 10 (5 males; 5 females)
Groups I to VI – Duration of exposure in each group was 69 hr.

For differential staining of sister chromatids, 80 µL bromodeoxyuridine (1 mg/mL distilled water) (Sigma-Aldrich Chemicals, St Louis, MO, USA) was added, mixed gently, and cultures were incubated at 37 °C for 72 hr in complete darkness. At the 69th hour, 30 µL of colchicine (1 mg/5 mL distilled water) (Sigma-
Aldrich Chemicals, St Louis, MO, USA) and 80 µL ethidium bromide (1 mg/mL distilled water) (Himedia, Mumbai, India) were added to cultures to arrest the cell division at metaphase stage. The cells were collected by centrifugation and then exposed to hypotonic solution (0.075 M KCl; pH 7) for 45 min and fixed in a mixture of acetic acid and methanol (1:3). Three changes of fixative were given prior to the preparation of the final cell suspension. A few drops of this concentrated cell suspension were placed on clean and chilled microslides and allowed to dry on a hotplate at 50 ºC.

The slides were stained according to the fluorescence-plus-Giemsa (FPG) method of Perry and Wolff16 for scoring of metaphase plates to analyse the sister chromatid exchange (SCE) and cell cycle proliferative index (CCPI).

**Analysis of sister chromatid exchanges (SCEs):** Metaphases in their second in vitro division (M₂) were selected for scoring on the basis of the spreading of chromosomes and differentiation of chromatids. At least 30 metaphases from each culture that were in their second in vitro division (M₂) were analysed for SCE. In these preparations cells dividing for the first (M₁), second (M₂), third or successive (M₃+) divisions in culture containing bromodeoxy uridine (BrdU) were designated by the differential staining pattern of sister chromatids. First division (M₁) cells contained chromosomes with both sister chromatids stained uniformly dark. Second division (M₂) cells contained only differentially stained chromatids with one chromatid darkly stained and its sister chromatid light stained, whereas, third or subsequent division (M₃+) cells had both sister chromatids stained lightly.

**Analysis of cell cycle kinetics (CCPI) or Replicative index (RI):** Differentially stained slides were scored for analysis of CCPI based on the staining pattern of chromosomes. At least 100 consecutive metaphases were analysed for each individual, classifying them as first (M₁), second (M₂), or third and subsequent (M₃+) generation cells. The CCPI for each individual was calculated according to the formula:

\[
\text{CCPI} = \frac{1 \times (M₁) + 2 \times (M₂) + 3 \times (M₃+)}{100}
\]

**Statistical analysis:** Results are expressed as means ± SE. The treated groups were compared with controls, and the antidote group was compared with combined treatment group by Student’s t test. Probability values of 0.05 or less were considered significant.

**RESULTS**

The SCE showed an increase in treated groups (Groups III–V) as compared to controls (Groups I and II) (Table 2). Reduction in the frequency of SCE was observed in Group VI in which ascorbic acid was added with fluoride and arsenic at ‘0’ hour as compared to the treated Groups III–V (Table 2).
Table 2. Frequency of sister chromatid exchange (SCEs) in lymphocyte cultures\textsuperscript{a}

<table>
<thead>
<tr>
<th>Group</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total M(_2)</td>
<td>Total SCEs</td>
<td>% SCE</td>
<td>Total M(_2)</td>
<td>Total SCEs</td>
<td>% SCE</td>
</tr>
<tr>
<td>I</td>
<td>136</td>
<td>644</td>
<td>473.53</td>
<td>140</td>
<td>624</td>
<td>445.72</td>
</tr>
<tr>
<td>II</td>
<td>135</td>
<td>591</td>
<td>437.78</td>
<td>130</td>
<td>559</td>
<td>430.00</td>
</tr>
<tr>
<td>III</td>
<td>127</td>
<td>660</td>
<td>519.69</td>
<td>131</td>
<td>659</td>
<td>503.05</td>
</tr>
<tr>
<td>IV</td>
<td>134</td>
<td>907</td>
<td>676.87</td>
<td>130</td>
<td>899</td>
<td>691.54</td>
</tr>
<tr>
<td>V</td>
<td>117</td>
<td>1103</td>
<td>942.74</td>
<td>109</td>
<td>1072</td>
<td>983.49</td>
</tr>
<tr>
<td>VI</td>
<td>146</td>
<td>695</td>
<td>451.37</td>
<td>128</td>
<td>671</td>
<td>524.22</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data are expressed as mean ± S.E. * P<0.05; † P<0.02; ‡ P<0.01; § P<0.001; no sign = Not significant.
Total metaphase counted in each individual of all groups = 100.
Comparisons between: Group I with Groups III or IV or V individually; Group V with Group VI.

Table 3. Frequency of SCE/cell, SCE/chromosome, and cell cycle proliferative index (CCPI) in lymphocyte cultures\textsuperscript{a}

<table>
<thead>
<tr>
<th>Group</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCE/Ce</td>
<td>SCE/Chrom</td>
<td>CCPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4.77±0.30</td>
<td>4.46±0.60</td>
<td>0.10±0.01</td>
<td>0.10± 0.004</td>
<td>1.48±0.02</td>
<td>1.46±0.02</td>
</tr>
<tr>
<td>II</td>
<td>4.37±0.08</td>
<td>4.30±0.11</td>
<td>0.10±0.04</td>
<td>0.09± 0.002</td>
<td>1.48±0.02</td>
<td>1.47±0.02</td>
</tr>
<tr>
<td>III</td>
<td>5.20±0.11</td>
<td>5.02±0.12†</td>
<td>0.11±0.02</td>
<td>0.11± 0.002†</td>
<td>1.40±0.02†</td>
<td>1.39±0.01‡</td>
</tr>
<tr>
<td>IV</td>
<td>6.80±0.22§</td>
<td>6.86±0.21§</td>
<td>0.15±0.01§</td>
<td>0.15± 0.005§</td>
<td>1.31±0.01§</td>
<td>1.33±0.02§</td>
</tr>
<tr>
<td>V</td>
<td>9.48±0.29§</td>
<td>9.85±0.18§</td>
<td>0.21±0.01§</td>
<td>0.22± 0.004§</td>
<td>1.28±0.01§</td>
<td>1.28±0.01§</td>
</tr>
<tr>
<td>VI</td>
<td>4.78±0.19§</td>
<td>5.24±0.10§</td>
<td>0.11±0.01§</td>
<td>0.12± 0.003§</td>
<td>1.46±0.01§</td>
<td>1.42±0.01§</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data are expressed as mean ± S.E. * P<0.05; † P<0.02; ‡ P<0.01; § P<0.001; no sign = Not significant.
Total metaphase counted in each individual of all groups = 100.
Comparisons between: Group I with Groups III or IV or V individually; Group V with Group VI.

The mean SCE/cell and SCE/chromosome ratios showed a nonsignificant increase in females but a significant increase (p<0.02) in males in the NaF treated Group III. A highly significant increase (p<0.001) occurred in both females and males in the As\(_2\)O\(_3\) and NaF + As\(_2\)O\(_3\) treated Groups IV and V as compared to the control Groups I and II. Group VI with ascorbic acid present showed a signif-
icant (p<0.001) decline in the frequency of SCE/cell and SCE/chromosome ratios as compared to Group V and were comparable to control values (Table 3).

The CCPI was significantly decreased in females (p<0.02) and in males (p<0.01) in the NaF and As₂O₃ treated Groups III and IV, while combined treatment with NaF and As₂O₃ (Group V) showed highly significant decline (P<0.001) in CCPI as compared to control Groups I and II. The CCPI showed a significant (P<0.001) increase in Group VI by addition of ascorbic acid, and the values were comparable to control (Table 3).

**DISCUSSION**

The DNA molecule is a target site of most, if not all, carcinogenic and mutagenic agents. Additionally, a number of agents like fluoride and arsenic are cytotoxic to the DNA molecule. Tsutsui et al. demonstrated that sodium fluoride induced an increase in SCEs in Syrian hamster embryo cells. Likewise, NaF + AlCl₃ induced an increase in SCEs in human lymphocytes, respectively, in culture. Some authors have reported an increase in the frequency of SCEs in fluoride endemic human populations of North Gujarat and South Gujarat, India, as compared to control populations and in peripheral blood lymphocyte cultures of volunteers having a concentration of 4–15 mg/L of fluoride in drinking water in China.

Zanzoni and Jung reported that addition of inorganic trivalent arsenic elevates the SCE rate in human lymphocyte cultures. Similarly, Burgdorf et al. observed an increase in the rate of SCEs in lymphocytes of patients treated with arsenic. Others found similar results, presumably mainly from exposure to fluoride, in lymphocytes of workers at a phosphate fertilizer factory in northern China and in workers of an aluminium factory. The results of the present study are in agreement with the above findings. However, Thompson et al. and Gadhia and Joseph however, observed no increase in SCE frequency or chromosomal aberrations in peripheral blood lymphocytes cultured with fluoride.

The cell cycle proliferative index declined when compared to the controls, in agreement with data of Sivokova and Dianovsky. He et al. reported that NaF and fluoroacetamide influence the cell cycle kinetics, chromosomal aberrations, and SCE frequencies in cultured red Muntjac cells. The percentage of M₁ cells increased, while that of M₂ and M₃ decreased significantly by NaF. In the present study, a significant lag in the cell cycle was observed which was similar to the foregoing report. Hayashi and Tsutsui also found that cytotoxicity and clastogenicity of NaF to cultured human diploid fibroblast are cell-cycle dependent, and cells in early and middle S phase are more sensitive to the effects of toxicant. Other workers have also reported cell mitotic delays.

Arsenic treatment likewise exhibits reduced mitotic activity and chromosomal alterations. The increase in SCE and lag in cell cycle proliferation suggests that fluoride and arsenic are genotoxic agents and may cause mutagenesis.

Fluoride may not cause direct damage to DNA. It could be indirectly involved in disruption of normal DNA replication. The actual mechanism could be quite
complex. The high electronegativity of the fluoride ion allows it to interact with both organic and inorganic compounds of cells, so its physiological effects could be expected to be more pronounced and varied than those caused by any other elemental ion. Thus fluoride has a great affinity for Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and phosphate, all of which are present in the culture medium and within the cells, so the active agent could be a magnesium-fluorophosphate, which is known to interfere with DNA polymerase and RNAase activities.\textsuperscript{31,32} Fluoride ion also forms a strong hydrogen bonds (NH---F–-) with purine and pyrimidine bases,\textsuperscript{33} which could disrupt the structures of both DNA and RNA and interfere with their synthesis or enhance the frequency of base-pair error during DNA replication. Mutagenic activity of NaF, KF, and NaHF\textsubscript{2} has also been demonstrated by Caspary \textit{et al}\textsuperscript{33} Similarly, chromosome aberrations were obtained in rat bone marrow cells treated with inorganic fluoride.\textsuperscript{34}

The biochemical mechanism by which arsenicals exert their chromosome damaging properties is not yet clear, but arsenic is known to interfere with several enzymic functions by blocking sulfhydryl groups,\textsuperscript{35} by replacing phosphorus in the phosphate group of DNA, and by forming a weak bond in the DNA chain.

Ascorbic acid (AA, vitamin C) has a role in maintaining the growth and integrity of leucocytes and has antioxidant and detoxification properties.\textsuperscript{36,37} In the present study, when AA was added to the cultures along with NaF and As\textsubscript{2}O\textsubscript{3} at ‘0’ hour, a significant protective effect by AA was manifested by decreasing the genotoxicity. Fluoride and arsenic alone and in combination induce genotoxicity in various cell lines, which was probably triggered by the generation of free radicals and increased lipid peroxidation \textit{in vitro}. Consequently, some chemotherapy approaches have been proposed that include the use of radical scavenging agents and antioxidant vitamins like vitamin C.\textsuperscript{38,39} Earlier studies from our laboratory have shown a protective effect of vitamin C on chemical induced toxicity \textit{in vitro} similar to the present study.\textsuperscript{14,20,39}

This work was presented at the XXth National Symposium on Reproductive Biology and Comparative Endocrinology, Department of Animal Science, Bharathidasan University, Tiruchirappalli, India, 7–9 January 2002. (Abstract No. RT-P-9, p. 94).

REFERENCES
Mitigation of F and As genotoxicity by ascorbic acid


