# AMELIORATION BY MELATONIN OF CHROMOSOMAL ANOMALIES INDUCED BY ARSENIC AND/OR FLUORIDE IN HUMAN BLOOD LYMPHOCYTE CULTURES[a]

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**SUMMARY: Standard cytochemical methods were used to investigate the ameliorative effect of melatonin (0.2 mM) on chromosomal aberrations in human lymphocyte cultures induced by arsenic ($As_2O_3$, 1.4 µM) and/or fluoride (NaF, 34 µM). $As_2O_3$ and/or NaF generated a significant increase in the incidence of chromosomal aberrations as compared to control levels. Combined treatment with $As_2O_3$ and NaF induced more chromosomal aberrations and aneuploidy than either reagent individually. Melatonin supplements brought about a significant decrease in the number of aberrations, with the percentage of amelioration varying between 53% and 88%. This reduction by melatonin of genotoxic effects exerted by As and/or F is probably attributable to its protective antioxidant action.**

Keywords: Arsenic and chromosomes; Chromosomal aberrations; Lymphocyte cultures; Melatonin and chromosomes; Fluoride and chromosomes; Oxidative stress.

## INTRODUCTION

Arsenic and fluoride are well-known water contaminants, and their toxicity on humans has been widely studied. Arsenic is a known human carcinogen and induces cancers of lung, skin, bladder, kidneys and liver.[1] It is introduced into water through dissolution of rocks, minerals, and ores, from industrial effluents including mining wastes, and from atmospheric deposition.[2] Inorganic $As^{+3}$ does not appear to be a mutagen in standard assays,[3] whereas $As^{+3}$ exposure *in vitro* produces chromosomal aberrations, DNA protein cross links, and SCE (sister chromatid exchanges) in hamster embryo cells[4,5] and in human lymphocytes.[6]

On the other hand, fluoride (F as $F^-$) is also known to be a major environmental contaminant. In India more than 20 states are affected by F toxicity, Gujarat being the most heavily impacted state.[7] With respect to genotoxic effects of F, there has been much confusion and a lack of adequate information. Joseph et al.[8] reported greater rates of SCE and chromosomal aberrations in people living in F-endemic villages than in nonendemic villages. Some authors, however, report that F does not induce genotoxic effects.[9] Earlier studies at our institution have reported mitigation of $As^{+3}$ and/or F genotoxicity by vitamins as antioxidants in the Indian population.[10]

Melatonin (N-acetyl-5-methoxytryptamine), the major secretory product of the pineal gland, is involved in the regulation of circadian rhythm and seasonal changes in vertebrate physiology.[11] It is identified as a powerful direct free radical scavenger[12] as well as an indirect antioxidant.[13] It has also been shown to reduce molecular damage effectively under conditions of elevated oxidative stress.[14] Melatonin, in fact, was found to exhibit better antioxidative properties under *in vitro* conditions than similar concentrations of α-tocopherol.[15] In view of its strong antioxidant activity, an investigation has now been undertaken of melatonin

[a]This paper is dedicated to our beloved teacher, the late Prof NJ Chinoy (d. 8 May 2006).

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for its ability to mitigate genotoxic effects of As and F in human blood cultures obtained from volunteers in India.

## MATERIALS AND METHODS

*Subjects:* Venous blood was collected in sterile heparinised syringes from healthy, consenting 20–25 year old non-smoking residents of Ahmedabad, India.

*Peripheral blood lymphocyte culture (PBLC):* PBLC was then performed by the method of Perry and Wolff.[16] To 7 mL of RPMI-1640 (HiMedia, Mumbai, pH 7.4) previously supplemented with 7% fetal calf serum (FCS), antibiotics (benzyl penicillin and streptomycin) and 100 µL of phytohemagglutinin [PHA], 5mg/5mL distilled water; Sigma-Aldrich, USA), nine drops of blood were added. After 48 hr of incubation, $As_2O_3$ (HiMedia, Mumbai) and NaF (Qualigens Fine Chemicals, Mumbai), with or without melatonin (HiMedia, Mumbai), were added. Harvesting was done after 24 hr.

Cultures were divided into eight groups. Group I was the control i.e., blood culture without any treatment. In Group II, lymphocytes were treated with As ($As_2O_3$; 1.4 µM), Group III with F (NaF; 34 µM), and Group IV with a combination of the same concentrations of $As_2O_3$ and NaF. Antioxidant Groups were melatonin (0.2 mM) supplemented with As (Group V), F (Group VI) and As+F (Group VII). Group VIII contained only melatonin added to the blood culture.

After 69 hr, 15 µL of colchicine (1 mg/5 mL distilled water, HiMedia, Mumbai) was added for 30 min to arrest cells at the metaphase stage. The pellet obtained after centrifugation was treated with hypotonic solution (0.075 M KCl) for 20 min at 37°C. These cells were then fixed by chilled 1:3 acetic acid:methanol fixative. Slides were prepared from the cell suspension obtained after two washes with fixative. These slides were then stained with 2% Geimsa stain and observed under the microscope at 100X for scoring structural and numerical aberrations.

*Analysis of parameters:* One hundred plates per group were analysed under the microscope for chromosome and chromatid breaks and gaps and number of chromosomes. Plates with a chromosome number greater than 44 were used for scoring structural and numerical aberrations. Percentage of amelioration was calculated by using the following formula:

$$\frac{\text{(Pro-oxidant Group – Respective Antioxidant Group)}}{\text{(Pro-oxidant Group – Control)}} \times 100$$

*Statistical analysis*: Results are expressed as mean ± SE. All the treated groups were compared with the control group, and the melatonin-supplemented groups were compared with their respective pro-oxidant groups by Student's t-test. P values less than 0.05 were considered to be significant.

## RESULTS

Addition of As (as $As_2O_3$) and/or F (as NaF) to the peripheral blood cultures showed a highly significant ($p<0.001$) increase in the number of total chromosomal aberrations as compared to the control cultures. On the other hand,

co-culturing of melatonin with As and/or F demonstrated a highly significant ($p<0.001$) decline in the number of induced chromosomal aberrations in comparison to the respective pro-oxidant group. Melatonin exhibited 52% and 74% amelioration from individual toxicity induced by As and F, respectively, and 64% amelioration from combined toxicity, for chromosomal aberrations (Table 1).

As seen in Table 2, the presence of As alone and in combination with F led to a very significant increase in the number of hypoploids ($p<0.001$) as compared to the control. F alone also caused a marked increase in the number of hypoploids ($p<0.05$). Addition of melatonin to the As and F treated cultures individually as well as to their combination produced significant reductions in the number of hypoploids with values that are comparable to those of the control culture. The amelioration was 79% and 88% from the toxicity of As and F, respectively ($p<0.05$ and $p<0.001$), and 84% from their combination ($p<0.001$). Melatonin alone had essentially no effect and gave readings comparable to the control values (Tables 1 and 2).

**Table 1.** Effect of melatonin (M) on As- and/or F-induced chromosome and chromatid breaks and gaps per 100 plates in human lymphocytes after 24-hr exposure for 5 individuals

| Group | Chromatids | | Chromosomes | | Mean[a] ± SE | Values in percent | t-test with control[b] | t-test with resp. Groups[c] | Amelioration (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Breaks | Gaps | Breaks | Gaps | | | | | |
| Control | 1.20 | 1.20 | 0.80 | 0.80 | 4.00 ± 0.80 | 100 | | | |
| As | 30.60 | 10.80 | 9.00 | 5.80 | 56.20 ± 1.57 | 1405 | 29.73* | | |
| F | 13.60 | 6.60 | 5.60 | 5.80 | 31.60 ± 0.96 | 790 | 22.21* | | |
| As+F | 34.80 | 15.60 | 7.60 | 6.80 | 64.80 ± 1.82 | 1620 | 25.31* | | |
| As+M | 15.20 | 5.60 | 4.80 | 3.20 | 28.80 ± 1.66 | 720 | 12.96* | 12.01* | 53 |
| F+M | 5.40 | 3.00 | 1.80 | 1.00 | 11.20 ± 1.07 | 280 | 3.41† | 14.20* | 74 |
| As+F+M | 14.60 | 3.60 | 4.60 | 3.20 | 26.00 ± 1.43 | 650 | 10.03* | 16.73* | 64 |
| M | 3.00 | 1.4 | 0.80 | 0.8 | 6.00 ± 0.56 | 150 | 1.65ns | | |

[a]Mean indicates total structural aberrations; [b]all groups were compared with control; [c]antioxidants groups were compared with respective pro-oxidant (As and/or F) group.
*p<0.001;†p<0.01; nsnot significant.

**Table 2.** Effect of melatonin on As and/or F induced numerical aberrations (hypoploidy) in human lymphocyte chromosomes after 24-hr exposure for 5 individuals

| Groups | Mean[a] ± SE | Values in percent | t-test with control[b] | t-test with resp.groups[c] | Amelioration (%) |
|---|---|---|---|---|---|
| Control | 11 ± 1.00 | 100 | | | |
| As | 23 ± 1.21 | 209 | 7.39* | | |
| F | 18 ± 1.12 | 164 | 4.27† | | |
| As+F | 26 ± 1.05 | 236 | 10.45* | | |
| As+M | 14 ± 1.05 | 127 | 1.65ns | 6.79* | 79 |
| F+M | 12 ± 0.89 | 109 | 0.60ns | 4.82* | 88 |
| As+F+M | 14 ± 1.00 | 127 | 1.70ns | 11.54* | 84 |
| M | 10 ± 1.03 | 90.9 | –0.70ns | | |

[a]Mean indicates number of numerical aberrations; ball groups were compared with control; cantioxidants groups were compared with respective pro-oxidant (As and/or F) group.
*p<0.001; †p<0.05, nsnot significant.

## DISCUSSION

Results of the present study revealed an increase in the number of structural as well as numerical chromosome and chromatid aberrations induced by As and/or F, indicating their genotoxicity. This induced genotoxicity is probably mediated by induction of oxidative stress and depletion of glutathione[17-19] as a result of the action of these two pro-oxidants. Electron spin resonance (ESR) analysis using

spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide also suggests that reactive oxygen species (ROS) participate in genotoxicity of As.[20] Thus, Oya et al.[21] found induction by As of chromosomal aberrations by hydrogen peroxide. Similarly, Eguchi et al.[22] observed increased frequency of chromosomal aberrations by As as observed here. Their study also revealed an increased frequency of aneuploidy on addition of As, in agreement with findings on HFW cells by Yih et al.[23] It has been documented that loss of p53 activity leads to aneuploidy along with gene amplification, and since As is known to alter p53 activity,[24] this might be the possible cause of the observed hypoploidy.

It is known that $F^-$ affects enzymatic activities, and this effect could delay mitotic cycles causing chromosomal breakages.[25] Similarly, reports are available that F increases chromosomal damage,[8,26,27] corroborating our data. Ardema et al.[28] proposed that NaF-induced aberrations might occur by an indirect mechanism involving inhibition of DNA synthesis/repair due to the formation of hydrogen bonds with nitrogen bases. Further, F also affects polymerase activity involved in DNA replication, since F has great affinity to $Ca^{+2}$, $Mg^{+2}$, and phosphate ions.[10] Chinoy et al.[29] also found increased aneuploidy in lymphocyte cultures after addition of NaF, again confirming the interaction of F with DNA nucleotides. In the present study, genotoxic effects of the combined treatment of As and F were more pronounced than with individual treatments, as might be expected. Likewise, genotoxic effects of As and/or F on human blood cultures of the Indian population have been reported by Nair et al.[10]

Melatonin has been shown to protect membrane lipids, nuclear DNA, and protein from oxidative damage induced by variety of free radical generating agents.[30,31,32] Vijayalaxmi et al.[33] reported that addition of melatonin reduced the incidence of primary DNA damage and chromosomal aberrations as well as the induction of micronuclei due to increased oxidative stress in irradiated human blood lymphocyte cultures. Melatonin, has also been used against oxidative stress-mediated DNA damage by chromium, cyclophosphamide, and hydrogen peroxide.[34,35] Since melatonin is subcellularly widespread, it might allow maximum interaction with all molecules thus protecting against ROS in both aqueous and lipid environment.[13] The present study also indicated a decrease in chromosomal aberrations on addition of melatonin to cell cultures exposed to pro-oxidant As and F. This ameliorative effect of melatonin could be attributed to scavenging hydroxyl radical, stimulating antioxidative enzymes, inhibiting pro-oxidative enzymes,[36,37] possibly by involvement in the formation of $As^{+3}/F^-$ complexes, thereby interfering with normal DNA and protein interactions, leading to chromosomal aberrations.

This study thus demonstrates that melatonin supplementation provides protection against chromosomal anomalies induced by As and/or F under *in vitro* condition

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