

EFFECTS OF MELATONIN AND AMLA ANTIOXIDANTS ON FLUORIDE-INDUCED GENOTOXICITY IN HUMAN PERIPHERAL BLOOD LYMPHOCYTE CELLS

Mandava V Rao,^{a,b} Swati B Thakur^a

Ahmedabad, India

SUMMARY: This investigation reports genotoxic effects of sodium fluoride (NaF) at 17, 34, and 51 μM for 72 hr and induction of sister chromatid exchanges (SCEs) and related changes, together with ameliorative effects by melatonin and amla, in human peripheral blood lymphocyte cell cultures. The cell cycle proliferative index (CCPI) significantly decreased with increasing F concentration. SCEs, average generation time (AGT), and population doubling time (PDT) also significantly increased with F exposure. Treatment with the antioxidants melatonin and amla, separately or in combination with the highest level NaF-treated group, showed a detectable but non-significant increase in CCPI and a decrease in SCEs, AGT, and PDT, comparable to levels in control cultures. The results indicate substantial amelioration of F genotoxicity in lymphocyte cells by melatonin, amla, and their combination.

Keywords: Amla; Cell cycle proliferation index; Fluoride genotoxicity; Human lymphocytes; Lymphocyte cell cultures; Melatonin; Sister chromatid exchanges.

INTRODUCTION

Water contamination and industrial pollution by fluoride (F) are widespread environmental problems, and acute or chronic exposure to high levels of F results in adverse health effects.¹ *In vivo* studies show that F induces nephrotoxicity,² reproductive toxicity,³ and neurotoxicity in humans as well as animals. Research in our laboratory on *in vitro* effects of F on human lymphocytes has shown F has genotoxic effects including increased chromosomal aberrations, micronucleus, and sister chromatid exchanges.^{4,5} By contrast, studies by others report that F has little or no *in vitro* genotoxic potential.⁶⁻⁸

In the course of our investigations, we found that melatonin has a significant protective effect against F toxicity.²⁻⁵ Melatonin is secreted from the pineal gland, mostly at nighttime, acting as a hormone functioning as a circadian diurnal rhythm mediator. It is also able to eliminate free radicals such as reactive oxygen species. Along with its by-products, it is also an extremely powerful antioxidant with a particular role in protecting DNA.⁹ Melatonin also has its own special antioxidative effect and is able to intensify the activity of endogenous antioxidative enzymes, which together exert a powerful antioxidative effect.^{10,11} However, the combination of F and melatonin with another antioxidant does not appear to have been reported.

Here, to undertake such an investigation, we added, besides melatonin, *Emblica officinales* (amla, or Indian gooseberry), which is a rich source of vitamin C (ascorbic acid) and polyphenols. Ascorbic acid is known for its antioxidant, anti-inflammatory, and antimutagenic properties, and it is a very effective free-radical

^aZoology Department, School of Sciences, Gujarat University, Ahmedabad 380 009, India; ^bFor correspondence: Dr MV Rao, Professor and Head, Zoology Department, School of Sciences, Gujarat University, Ahmedabad 380 009, India. E-mail: zooldeptgu@satyam.net.in

scavenger.¹² However, there are some *in vivo* studies indicating that the antioxidant activities of amla cannot be attributed to ascorbic acid alone and that the overall effect is also due to the presence of polyphenols such as ellagic acid, gallic acid, and various tannins.¹³

After reviewing the properties of melatonin and amla, we set about to investigate their ameliorating effects, alone and in combination, on the genotoxicity of F acting on human blood cells in *in vitro*.

MATERIALS AND METHODS

Sample Collection: Peripheral blood samples, which were then heparinised, were collected from healthy, non-smoking, non-F affected individuals between 20 and 25 years of age with their consent.

Peripheral blood lymphocyte cultures: Using the standard protocol,¹⁴ 7 mL RPMI-1640 media (Himedia, Mumbai, pH 7.4), pre-supplemented with 7% fetal bovine serum (Himedia, Mumbai), was placed in the culture tube. To this mixture, 0.1 mL of phytohemagglutinin (PHA, 1 mg/mL, Sigma-Aldrich, USA) and 0.5 ml of heparinised blood was added. For the cultures of sister chromatid exchange 80 μ L of bromodeoxyuridine (BrdU, 1 mg/mL, Sigma-Aldrich, USA) was added in the dark for further fluorescent staining.¹⁵ Cultures were divided into 11 groups with addition of test chemicals at the 0th hour (Table 1).

Table 1. Experimental Groups

Group	Treatment
I	Control
II	NaF low-dose (LD) (17 μ M)
III	NaF mid-dose (MD) (34 μ M)
IV	NaF high-dose (HD) (51 μ M)
V	Melatonin alone (0.2 mM)
VI	Amla alone (20 μ g)
VII	Melatonin (0.2 mM) + amla (20 μ g)
VIII	NaF high-dose (51 μ M) + melatonin (0.2 mM)
IX	NaF high-dose (51 μ M) + amla (20 μ g)
X	NaF high-dose (51 μ M) + melatonin (0.2 mM) + amla (20 μ g)
XI	Positive control (EMS) ^a (1.93 mM)

^a Ethylmethane sulphonate.

Chemicals used were sodium fluoride (NaF, Qualigen fine chemicals, Mumbai), Melatonin (Himedia, Mumbai), amla (Himalaya Drug Company, Bangalore), and ethylmethane sulphonate (EMS, 1 mg/mL, Sigma-Aldrich, USA). The cultures were incubated at 37°C for 69 hr.

At the 69th hr, 20 μ L of colchicine (1 mg/5 mL, Himedia, Mumbai) was added to the culture tubes and incubated for 1 hr at 37°C. The tubes were then centrifuged at

2000 rpm for 15 min, and the supernatant was discarded to obtain the pellet. To the pellet 5 mL of hypotonic solution (0.075 M potassium chloride, Merck, Germany) was added, and the mixture was incubated for 15 min at 37°C in a waterbath. The cells were then fixed with chilled fixative (1:3 acetic acid:methanol) and centrifuged again. After 2 to 3 washes with fixative to clear the pellet, slides were prepared from the final suspension.

Staining procedure: The fluorescent-giemsa stain method¹⁵ was used to score sister chromatid exchanges (SCEs) and different metaphase plates (M1, M2, and M3) to follow the three successive divisions. These slides were observed under higher magnification.

Analysis of parameters: A total of 100 metaphase plates per culture were analysed to score SCEs and differentially stained metaphase plates (M1, M2 and M3). The cell cycle proliferating index (CCPI) was calculated by following formula:

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

Where, M1 = first mitotic division, M2 = second mitotic division, and M3 = third mitotic division.

For calculating SCEs per plate and per chromosome, and for calculating average generation time (AGT) and population doubling time (PDT), the following formula was used:

$$\text{SCE/Plate} = \frac{\text{Total SCEs scored}}{\text{Total M2 plate}}$$

$$\text{SCE/Chromosome} = \frac{\text{Total SCEs scored}}{\text{SCE/Plate}}$$

$$\text{AGT} = \frac{72 \text{ hr (BrdU time)}}{\text{CCPI}}$$

$$\text{PDT} = \frac{24 \text{ hr (1 cycle = 24 hr)}}{\text{CCPI}}$$

Statistical analysis: Percentage (%) amelioration was calculated using the following formula:⁴

$$\text{Percentage amelioration} = \frac{(\text{Pro-oxidant group} - \text{antioxidant group}) \times 100}{\text{Control} - \text{Pro-oxidant group}}$$

The data were analyzed statistically using Student's "t" test and Analysis of Variance (ANOVA). A level of $p < 0.05$ was accepted as significant.

RESULTS

Sister chromatid exchanges (SCEs): As seen in Table 2, SCEs/plate and SCEs/chromosome showed a non-significant increase in the low- and mid-dose NaF exposure groups, whereas the high-dose (HD) group exhibited a significant increase in SCEs per metaphase plate ($p < 0.001$) and SCEs per chromosome ($p < 0.05$). On the other hand, co-culturing the HD group with the antioxidants melatonin and amla gave a highly significant reduction in these ratios.

Table 2. Percentage of M1, M2, M3 plates and frequency of sister chromatid exchanges (SCEs) per 100 metaphase plates

Group	M1 Plate	M2 Plate	M3 Plate	Exchanges	SCEs/Plate	SCEs/Chromosome
I	33.6 ± 0.51	48 ± 0.45	18.4 ± 0.51	157 ± 1.00	3.272 ± 0.026	0.068 ± 0.001
II	38.6 ± 0.40	49 ± 0.32	12.4 ± 0.24	166.4 ± 0.60	3.396 ± 0.014 ^{ns}	0.069 ± 0.001 ^{ns}
III	42.8 ± 0.97	47.2 ± 0.73	10 ± 0.63	169.4 ± 0.40	3.592 ± 0.053 ^{ns}	0.076 ± 0.002 ^{ns}
IV	48.2 ± 0.66	44.6 ± 0.40	7.2 ± 1.02	182 ± 2.21	4.083 ± 0.078 [†]	0.092 ± 0.002 [*]
V	35.8 ± 0.37	49 ± 0.45	15.2 ± 0.37	159.2 ± 0.73	3.250 ± 0.039 ^{ns}	0.066 ± 0.001 ^{ns}
VI	35.2 ± 0.97	49.8 ± 1.20	15 ± 0.55	157 ± 1.67	3.160 ± 0.084 ^{ns}	0.064 ± 0.003 ^{ns}
VII	35.2 ± 0.73	49 ± 0.77	15.8 ± 0.66	159.6 ± 1.29	3.260 ± 0.048 ^{ns}	0.067 ± 0.002 ^{ns}
VIII	36 ± 1.05	50 ± 1.14	14 ± 0.45	160.8 ± 1.07	3.222 ± 0.070 ^{ns}	0.065 ± 0.003 ^{ns}
IX	35.6 ± 1.29	50.8 ± 1.07	13.6 ± 0.40	161.6 ± 1.12	3.188 ± 0.083 ^{ns}	0.063 ± 0.003 ^{ns}
X	35 ± 0.63	50 ± 0.55	15 ± 0.55	158.2 ± 1.07	3.166 ± 0.041 ^{ns}	0.063 ± 0.001 ^{ns}
XI	61.8 ± 1.62	34.8 ± 1.36	3.4 ± 0.6	197.2 ± 1.07	5.705 ± 0.247 [†]	0.166 ± 0.015 [†]

Values are expressed as mean ± SEM. All groups are compared with the control group I using Student's t-test. * $p < 0.05$, [†] $p < 0.001$, ns = non significant.

Cell cycle proliferative index (CCPI): This index showed a highly significant decrease ($p < 0.001$) in a dose-dependent manner in the three NaF-dose groups II, III, and IV (Table 3). When the high-dose (HD) NaF group was co-treated with melatonin and amla and their combination, the culture showed significant increase in the melatonin and amla alone in the supplemented HD NaF groups VIII and IX with an ameliorative rate averaging 76.92% and 80.61%, respectively. With melatonin and amla together in group X, the ameliorative rate was 84.61%.

Average generation time and population doubling time: Average generation time (AGT) and population doubling time (PDT) revealed a significant increase at all three F doses of F. In the cell cultures supplemented with melatonin alone, amla alone, and the combination of both in groups V, VI, and VII, respectively, these indices were comparable to those in the control group I (Table 3). These results are confirmed by the amelioration percentages.

Table 3. Table showing cell cycle proliferating index (CCPI), average generation time (AGT), and population doubling time (PDT) per 100 plates.

Group	CCPI	% Amelioration	AGT	% Amelioration	PDT	% Amelioration
I	1.85 ± 0.01		38.96 ± 0.19		12.99 ± 0.06	
II	1.74 ± 0.01 †		41.43 ± 0.14†		13.81 ± 0.05†	
III	1.67 ± 0.01 †		43.08 ± 0.38†		14.36 ± 0.13†	
IV	1.59 ± 0.02 †		45.30 ± 0.48†		15.10 ± 0.16†	
V	1.79 ± 0.01 ^{ns}		40.14 ± 0.14 ^{ns}		13.38 ± 0.05 ^{ns}	
VI	1.80 ± 0.01 ^{ns}		40.05 ± 0.23 ^{ns}		13.35 ± 0.08 ^{ns}	
VII	1.81 ± 0.01 ^{ns}		39.87 ± 0.26 ^{ns}		13.29 ± 0.09 ^{ns}	
VIII	1.78 ± 0.01 [*]	76.92%	40.46 ± 0.26 ^{ns}	81.38%	13.49 ± 0.09 ^{ns}	81.51%
IX	1.78 ± 0.02 [*]	80.76%	40.46 ± 0.36 ^{ns}	82.80%	13.49 ± 0.12 ^{ns}	82.93%
X	1.80 ± 0.01 ^{ns}	84.61%	40.01 ± 0.24 ^{ns}	85.64%	13.34 ± 0.08 ^{ns}	85.78%
XI	1.42 ± 0.02 †		50.89 ± 0.76†		16.96 ± 0.25†	

Values are expressed as mean ± SEM. All groups are compared with the control group I using student's t-test. *p<0.05, †p<0.001, ns = non-significant.

DISCUSSION

The present study confirmed the genotoxicity of F in human blood cell cultures *in vitro*. This effect of F on cellular metabolism and physiology varies accordingly to the cell type, concentration, and time of exposure.¹⁶ F affects the enzyme activities of the cell by binding with their protein OH groups and increases the oxidative stress.¹⁷ Zhang et al.¹⁸ reported cell cycle delay in rat osteoblasts. As found in our studies, it has been suggested that F can bind to Ran protein,¹⁹ which regulates nuclear cytoplasmic transport during G1, S and G2 phases and is implicated in the arrangement of microtubules due to which the cell cycle kinetics decrease in accordance to our results.

Sister chromatid exchanges (SCEs) occur during DNA replication as cells pass to S phase.²⁰ Exposure to radioactive and genotoxic agents may affect the cell cycle and increases the frequency of SCEs, AGT and PDT.²¹⁻²³ Our findings indicated F affected the cell cycle, cell membrane and protein, and led to DNA damage with an increase in SCE frequency and decreased CCPI. In addition, our study is also supported by the report that SCEs/cell significantly increased in phosphate fertilizer factory employees exposed to high concentration of HF and SiF₄ compared to normal controls.²⁴

In regard to melatonin and amla, their antioxidant and free radical scavenging properties have long been recognized.¹¹ Although melatonin is a highly effective

scavenger of a variety of toxic radicals,⁹ its metabolites resulting from interaction with free radicals are also proficient in neutralising damaging reactants.²⁵ In accord with the findings presented here, melatonin has been shown to be a powerful protective agent against toxic effects of radioactive and toxic agents on human lymphocyte cultures.^{26,27} Similarly, amla, the other supplement studied here, also shows a strong antioxidant cascade mechanism that is partially due to the presence of ascorbic acid and phenolic compounds.^{12,13,28} Amla has also proven itself as a protective agent against cadmium toxicity in bone marrow chromosomes of Swiss albino mice in support of the present investigation.²⁹ Additionally, melatonin has also been shown to enhance the beneficial biological effects.^{30,31} Since amla is a rich source of ascorbic acid, it is plausible that their combined effect shows better results, as in the present study, than either one alone.

From our findings, we conclude that melatonin and amla and their combination have ameliorative effects against F-induced genotoxicity, making the cell cycle kinetics and SCEs frequency comparable to those in control cultures. Although beneficial individually, the combination of melatonin and amla was more effective in mitigating the genotoxic potential of F.

ACKNOWLEDGEMENTS

This work is supported by Research Grants from Gujarat State Biotechnology Mission (GSBTM), Gandhinagar and Clinic Center of Gujarat University, Ahmedabad, India.

REFERENCES

- 1 Fawell J, Bailey K, Chilton J, Dahi E, Fewtrell L, Magara Y. Fluoride in drinking-water. London: IWA Publishing on behalf of the World Health Organization; 2006.
- 2 Rao MV, Chawla SL, Patel N. Melatonin reduction of fluoride-induced nephrotoxicity in mice. *Fluoride* 2009;42(2):110-6.
- 3 Rao MV, Bhatt RN. Protective effect of melatonin on fluoride-induced oxidative stress and testicular dysfunction in rats. *Fluoride* 2012;45(2):116-24.
- 4 Rao MV, Tiwari H. Amelioration by melatonin of chromosomal anomalies induced by arsenic and /or fluoride in human blood lymphocyte cultures. *Fluoride* 2006;39(4):255-60.
- 5 Pant H, Rao MV. *In vitro* melatonin supplementation against genetic toxicity by arsenic and fluoride. *Adv Biores* 2010;1(2):17-24.
- 6 Li Y, Liang CK, Katz BP, Brizendine EJ, Stooky GK. Long-term exposure to fluoride in drinking water and sister chromatid exchange frequency in human blood lymphocytes. *J Dent Res* 1995;74(8):1468-74.
- 7 Slamenová D, Gábelová A, Ruppová K. Cytotoxicity and genotoxicity testing of sodium fluoride on Chinese hamster V79 cells and human EUE cells. *Mutat Res* 1992;279:109-15.
- 8 Thomson EJ, Kilanowski FM, Perry PE. The effect of fluoride on chromosome aberration and sister-chromatid exchange frequencies in cultured human lymphocytes. *Mutat Res* 1985;144:89-92.
- 9 Reiter RJ, Kormaz A, Paredes SD, Manchester LC, Tan DX. Melatonin reduces oxidative/nitrosative stress due to drugs, toxins, metals, and herbicides. *Neuro Endocrinol Lett* 2008;29(5):609-13.
- 10 Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci* 2000;7:444-58.
- 11 Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: a potent endogenous hydroxyl radical scavenger. *Endocr J* 1993;1:57-60.

- 12 Ghosh A, Sharma A, Talukdar G. Relative protection given by extract of *Phyllanthus emblica* fruit and an equivalent amount of vitamin C against a known clastogen— caesium chloride. *Food Chem Toxicol* 1992;30:865-9.
- 13 Bhattacharya AA, Chatterjee A, Ghosal S, Bhattacharya SK. Antioxidant activity of active tannoid principles of *Embllica officinalis* (amla). *Indian J Exp Biol* 1999;37:676-80.
- 14 Hungerford DA. Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosome by treatment with hypotonic KCl. *Stain Technol* 1965;40:333-8.
- 15 Perry P, Wolff S. New Geimsa method for the differential staining of sister chromatids. *Nature* 1974;251:156-8.
- 16 Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. *Chem Biol Interact* 2010;188 (2):319-33.
- 17 Adamek E, Powlowska-Góral K, Bober K. *In vitro* and *in vivo* effects of fluoride ions on enzyme activity. *Ann Acad Med Stetin* 2005;51:69-85. [in English and Polish].
- 18 Zhang Y, Sun G, Jin Y, Wang Y. Effects of fluoride on cell cycle and apoptosis in cultured osteoblasts of rats. *Wei Sheng Yan Jiu* 2003;32(5):432-3. [in Chinese].
- 19 Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol Rev* 2001; 81:153-208.
- 20 Wolff S, Bodycote J, Painter RB. Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation of different stages of the cell cycle: the necessity for cells to pass through S. *Mutat Res* 1974;25:73-81.
- 21 Wilson DM 3rd, Thompson LH. Molecular mechanisms of sister-chromatid exchange. *Mutat Res* 2007;616:11-23.
- 22 Agalakova NI, Gusev GP. Molecular mechanisms of cytotoxicity and apoptosis Induced by inorganic fluoride [review]. *ISRN Cell Biology* 2012; Article ID 403835, 16 pages, doi:10.5402/2012/403835. Available from: <http://www.hindawi.com/ism/cb/2012/403835/> [Abstract in *Fluoride* 2012;45(2):145].
- 23 Pant HH, Rao MV. Evaluation of *in vitro* anti-genotoxic potential of melatonin against arsenic and fluoride in human blood cultures. *Ecotoxicol Environ Saf* 2010;73(6):1333-7.
- 24 Meng Z, Meng H, Cao X. Sister-chromatid exchanges in lymphocytes of workers at a phosphate fertilizer factory. *Mutat Res* 1995;334(2):243-6.
- 25 Reiter RJ, Paredes SD, Manchester LC, Tan DX. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. *Crit Rev Biochem Mol Bio* 2009;44(4):175-200.
- 26 Lialiaris T, Lyrtzopoulos E, Papachristou F, Simopoulou M, Mourelatos C, Nikolettos N. Supplementation of melatonin protects human lymphocytes *in vitro* from the genotoxic activity of melphalan. *Mutagenesis* 2008;23:347-54.
- 27 El-Sokkary GH. An autoradiographic study of cellular proliferaton, DNA synthesis and cell cycle variability in the rat liver caused by phenobarbital-induced oxidative stress: the protective role of melatonin. *Cell Mol Biol Lett* 2007;12(3):317-30.
- 28 Kumar GS, Nayaka H, Dharmesh SM, Salimath PV. Free and bound phenolic antioxidants in amla (*Embllica officinalis*) and turmeric (*Curcuma longa*). *Journal of Food Composition and Analysis* 2006;19:446-52.
- 29 Singh P, Chaudhary S, Patni A, Sankhla V. Effect of cadmium chloride induced genotoxicity in bone marrow chromosomes of Swiss albino mice and subsequent protective effects of *Embllica officinalis* and vitamin C. *Journal of Herbal Medicine and Toxicology* 2007;1(2):67-71.
- 30 Reiter RJ. Melatonin: clinical relevance. *Best Pract Res Clin Endocrinol Metab* 2003;17:273-85.
- 31 Gitto E, Tan DX, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, et al. Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. *J Pharm Pharmacol* 2001;53:1393-401.