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RESEARCH PAPER

In vitro Melatonin Supplementation against Genetic toxicity By Arsenic and Fluoride

Pant, H.* and Rao, M. V.
*P D Patel institute of Applied Sciences, Charotat University of Science and Technology (CHARUSAT), At and PO Petlad, Dist Anand - 388 421, INDIA.
Zoology Department, University School of Sciences, Gujarat University, Navrangpura-380 009, Ahmedabad, INDIA
E mail: hemlata.pant@gmail.com zooldeptgu@satyam.net.in

ABSTRACT
The present study was conducted to elucidate protective effect of melatonin on arsenic and fluoride induced genotoxicity, in human blood cultures. It contained three major groups namely controls group, pro-oxidants group and anti-oxidants group with 24 hr exposure. Controls group included untreated control, positive control (Ethyl methane sulphonate, 1.93mM) and melatonin control (0.2mM). Prooxidants group contained cultures exposed to arsenic (1.4µM) and fluoride (34µM), alone and in combination. Antioxidants group consisted above mentioned prooxidants along with melatonin to assess the ameliorative action. Genetic damage indices studied were chromosomal aberrations (both structural and numerical), micronuclei with Cytokinesis-Block-Proliferative-Index and primary DNA damage elucidated through comet assay. A comparison of all the groups with untreated control and prooxidants group with respective anti-oxidants group evaluated the effect of melatonin. Melatonin led to significant decrease in the genotoxic indices induced by in vitro exposure of arsenic and fluoride, resulting in more than 50 percent amelioration. Results from the present study would set up a base to design further in vivo studies for searching an antidote to fight against these water contaminants.
***Key Words*:** Arsenic; fluoride; chromosomal aberrations; micronuclei; melatonin; mitigation; comet assay.

INTRODUCTION

Arsenic (As) contamination from the leaching of mine deposits and emissions from industries have been reported in countries like USA, Japan, UK, Australia, while As in natural aquifers used for drinking water supply has affected millions of people in India, Bangladesh, China, Chile, Hungary, Mexico, Argentina, Thailand and Taiwan [1]. With respect to human health impact, the situation is serious in West Bengal (India) and Bangladesh. Mahata et al [2] reported an enhanced frequency of micronuclei (MN) in lymphocytes, oral mucosa and urothelial cells and chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in lymphocytes of individuals exposed to As through drinking water in West Bengal. *In vitro* study of As on human lymphocytes also showed genotoxic effects like, increased CAs, MN, and SCEs[3,4].Moreover Bau et al [5] confirmed that As induced double strand break can come from either nitric oxide or reactive oxygen species (ROS) or both depending on the cell type. In addition, increased As concentration showed a positive correlation with plasma ROS levels and an inverse correlation with plasma antioxidant level,6 supported by the investigations revealing the activation of NADH oxidase in the presence of As to produce superoxide, which then cause oxidative DNA damage[7].

Another prooxidant of the present study is fluoride (F). F is a ubiquitous substance found naturally in food and water and also as a common air pollutant in some industrial productions. Till now there have been many conflicting reports on the genotoxic potential of F. Some reports suggested that F exhibits chromosomal damage in human cells, on the contrary, many studies report that F has no genotoxic potential [8,9,10]. F is known to stimulate the respiratory burst and the production of superoxide radical in human, guinea pig and rabbit neutrophils as well as in human polymorphonuclear leukocytes [11]. Further supporting the findings, Chlubek [12] provided evidence for increased production of OH and $.O_2$- radicals with the increase in F concentration, accompanied with the decrease in the activity of free radical scavenging enzymes (SOD, GSH-Px). The activity of free

radical scavenging enzymes were also found to be decreased in people living in areas of endemic fluorosis, supporting the ROS generation by fluoride, which probably induce genotoxicity [13]. Thus our aim was to search for a potent antioxidant against both As and F for a mitigating effect.

Melatonin is N-acetyl 5 methoxytryptamine, which is a mammalian hormone synthesized mainly in the pineal gland. This pineal hormone has been shown to exhibit distinct antioxidant features, *in vitro* and *in vivo*. It is a powerful antioxidant, effective in both aqueous and lipid phases. It is able to cross the blood brain barrier thus it has an easy access to every cell and its compartments, which makes it an effective antidote against ROS induced damage [14] Tan et al [15] documented that the ability of melatonin to neutralize the highly toxic hydroxyl radical is more efficient than reduced glutathione as well as mannitol indicating its role in inhibiting lipid peroxidation. Further the study by Vijayalaxmi et al [16] analysed the effect of melatonin upon UV radiation induced DNA damage and revealed that it not only scavenges radiation induced hydroxyl radicals but also influences the simultaneous activation of enzymes involved in repair of lesions in cellular DNA.

Till now melatonin has not been investigated as an antigenotoxic agent against As and F and since both these environmental pollutants exert their effect by producing oxidative stress, melatonin becomes a suitable candidate for ameliorative studies. Taking all this into account the antioxidant and ROS scavenging property of melatonin was explored against the *in vitro* genotoxicity induce by As and F.

MATERIALS AND METHODS

Chemicals

Arsenic trioxide (As2O3) was obtained from HiMedia Lab, Mumbai, 2.5mM stock solution of As2O3 was prepared in dilute alkali and sterilized with 0.22µ filter. The working solution was prepared by diluting the stock 100 times with sterilized double distilled water (DDW). Sodium fluoride was obtained from Qualigens fine chemicals, Mumbai, India. A 5mg/ml solution was prepared in DDW and then sterilized by passing through 0.22µ filter. Melatonin was procured from HiMedia Lab, Mumbai, India and a 40mM solution was prepared in 1:1;dimethyl sulfoxide: DDW solution. Ethyl methane sulphonate was obtained from HiMedia Lab, Mumbai, India, 240mg/ml solution prepared in dimethyl sulfoxide. The details of other chemicals is as follows: RPMI-1640, fetal calf serum, colchicine (HiMedia Lab.Mumbai, India), streptomycin from Sarabhai Piramal pharma Pvt Ltd, Vadodara, India and penicillin from Alembic Ltd, Vadodara, India. Phytohaemaglutinin (PHA), sodium lauryl sarcosinate and cytochalasin B were obtained from Sigma-Aldrich chemicals pvt ltd, St. Louis, MO.

Experimental design

The study included three major groups: prooxidants group, antioxidants group and controls, all in duplicates. Prooxidants group consisted three subgroups: As, F and the combination of As and F (As+F). Similarly antioxidants group represented three subgroups wherein melatonin was cocultured with the prooxidants; denoted as As+M, F+M and As+F+M. Controls group consisted a negative control without any treatment, a melatonin control wherein melatonin alone was added to the cultures and a positive control where ethyl methane sulphonate (EMS), a known mutagen, was added to blood cultures (Table I). The exposure time for all treatments was 24 hrs.

Table I: Shows different subgroups along with the denotation and treatment dose present in the experimental groups of the study

Experimental Groups	Treatment	Doses
Controls group	Control (Con)	
	Ethyl methane sulphonate (EMS)	EMS(1.93mM)
	Melatonin (M)	Mel(0.2Mm)
Prooxidants group	Arsenic (As)	As_2O_3(1.4µM)
	Fluoride (F)	NaF(34µM)
	Arsenic + Fluoride (As+F)	As_2O_3(1.4µM)+NaF:(34µM)
Antioxidants group	Arsenic + Melatonin (As+M)	As_2O_3(1.4µM)+Mel(0.2mM)
	Fluoride + Melatonin (F+M)	NaF(34µM)+ Mel(0.2mM)
	Arsenic + Fluoride + Melatonin (As+F+M)	As_2O_3(1.4µM)+NaF(34µM)+ Mel(0.2mM)

Sample collection

Consent forms were duly signed by the volunteers before blood collection following the ethical guidelines by ICMR, India. 10ml blood was collected from 15 normal healthy individuals for each set of experiment, within the age group of 20-30 years in heparinised vacuutainers (Greiner bio-one, Austria). The volunteers were from Ahmedabad region of Gujarat state in India, without any addiction and exposure to As or F through water.

Peripheral blood lymphocyte culture (PBLC)

Five hundred micro liter Peripheral blood was cultured [17] in 7ml RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, 100mg/L streptomycin, 100units/ml penicillin and 100 µg (0.1ml of 1mg/ml) PHA. These closed cultures were incubated at 37°C for 45 hrs and then the *in vitro* treatment was given for 24 hrs. 1.4µM of As_2O_3 working solution, 34µM of NaF, 0.2mM of melatonin was added according to the groups divided.

Chromosomal aberrations

Harvesting: At 69th hr, after culture set up, colchicine (20µl of 1mg/ml) was added to 7ml culture and centrifuged after 30 minutes. The pellet formed was incubated, at 37°C, with hypotonic solution (0.075M KCl) for 20 minutes, and then fixed with fixative (1:3 acetomethanol). Final cell suspension was made after two washes with fixative, and 3 to 4 drops of this suspension was added to wet chilled slides and flamed.

*Analysis:*Slides were labelled and stained with 2% giemsa for analysis [18] Chromosomal aberrations can either be structural or numerical. Numerical aberrations were analysed by counting chromosome number in hundred metaphase plates from each group. Plates with chromosome number less than 44 were not included in the analysis.

Structural aberrations include breaks and gaps. If the unstained region on the chromosome was more than its diameter then it was considered as break and if it was less than the diameter then it was included in gaps. Break on single chromatid was termed as chromatid break and on both the chromatids at the same loci was termed as chromosome break. Similar rules were applied to chromatid breaks and gaps. Slides were scanned for 100 plates per group.

Cytokinesis Block Micronuclei Assay (CBMN)

Assay was performed with slight modifications in the standard method [19] Lymphocyte cultures were set and doses were added at 45th hr as mentioned above. At 69th hr cytochalasin B (30µl of 1mg/ml) was added and kept for 24 hr incubation. Cultures were then centrifuged and pellet was suspended in 5ml hypotonic solution (0.075M KCl) for 10 minutes and then fixed in 1:3 acetomethanol, then kept in icebath for 10 minutes. Final suspension was prepared after gently flushing the cultures and washing once with fixative. 4 to 5 drops of suspension was added to wet chilled slides and air dried on hot plate.

*Analysis:*Slides were stained in 4% giemsa and scanned for micronuclei in 1000 binucleates in each group.

*Analysis of CBPI:*Since micronucleus expression is dependent on cell proliferation, quantification of cell proliferation and cell death should be carried out to obtain a sound evaluation of cell kinetics and micronucleus frequencies. In studies with cytochalasin B, determining the cytokinesis-block proliferation index (CBPI) is important since it indicates the number of cell cycles per cell during the period of exposure to cytochalasin B [20].

$$CBPI = \frac{M1 + (2 \times M2) + (3 \times M3)}{\text{Total number of cells}}$$

Where, **M1** is the number of mononucleate cells, **M2** is the number of binucleate cells and **M3** is the number of multinucleate cells.

Comet Assay

Base slides were prepared by dipping them in 1% normal melting point agarose (NMA) (500mg/50ml DDW), wiped from the back side and dried at room temperature. The lymphocyte cultures were then centrifuged and the pellet was suspended in 0.5 ml calcium magnesium free phosphate buffer saline (PBS). Twenty micro liter of this cell suspension was added to 80µl of 0.75% low melting point agarose, mixed properly and added to the base slide. After covering it with coverslip the slide was refrigerated for 10-15 min. 80µl of low melting agarose (without cells) was then layered on the slide and kept in refrigerator after replacing the coverslip. The slides were put into cold lysing solution [2.5

M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0), 1% sodium lauryl sarcosinate along with fresh addition of 1% Triton X-100and 10% DMSO], in dark at 4ºC for 16-18 hrs. The slides were then washed with DDW and electrophoresed after 40 mins incubation in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 30 min, at 25 V, 300 mA. Slides were neutralized by washes with 0.4 M Tris, pH 7.5, placed into 100% ethanol and then air-dried [21].

Analysis: Slides were stained with 0.02 mg/ml ethidium bromide (Sigma). Comet tail lengths were scored (50cells/slide) under an Axiophot Microscope (Zeiss) by using the Comet score software. All measurements were done on duplicate slides.

Statistical analysis

Percentage amelioration was calculated by using the following formula [3]:

$$\text{Percentage amelioration} = \frac{\text{(Pro oxidant group-Antioxidant group) X100}}{\text{Control- Pro Oxidant Group}}$$

The significance levels were determined by students t-test wherein, values for all the groups were compared to control group. Also each of prooxidants group was compared with the respective antioxidant groups. $p<0.01$ was considered as the significance limit.

The data was also analyzed by one way ANOVA to analyze the significant effect of the treatments, since more than two groups are present.

RESULTS

Structural aberrations

Total aberrations induced by As and F, individually as well as in combination were significantly high compared to untreated control group values. Melatonin co cultured with the prooxidants, in all the groups, brought about remarkable decrease in the induced structural aberrations, in comparison to the respective prooxidant groups. When compared to control, values for melatonin along with F were comparable, while for melatonin with arsenic and the combination of As and F demonstrated significant increase in chromosomal anomalies. The percentage amelioration calculated for all groups varied from 52% to 73%, melatonin against F showing maximum mitigation (Table II).

Table II: Human peripheral blood was cultured and was exposed *in vitro* to different treatment groups, as mentioned in the table, for 24 hrs to analyze the genotoxic indices such as micronuclei, CBPI, structural and numerical aberrations. This table indicates frequency of MN induction, structural aberration and numerical aberrations along with CBPI in all the groups. Percent amelioration due to melatonin is calculated for groups where melatonin is co-cultured with prooxidants.

Groups	Micronuclei		CBMI	Structural aberrations		Numerical Aberrations	
	Mean±SE	%Amelioration	Mean±SE	Mean±SE	%Amelioration	Mean±SE	%Amelioration
Control	8.20±0.44		1.42±0.03	4.00±1.41		9±0.49	
EMS	27.00±0.89***		1.20±0.01***	72.80±3.25***		17±1.49***	
M	8.40±0.46^{NS}		1.41±0.03^{NS}	6.00±0.71^{NS}		8±0.66^{NS}	
As	28.60±2.52***		1.29±0.03*	56.20±5.48***		21±1.85***	
F	21.20±1.73***		1.30±0.05^{NS}	56.20±2.04***		16±1.17***	
As+F	33.20±3.12***		1.26±0.04**	64.80±7.42***		24±0.75***	
As+M	12.60±1.64* (###)	78.43	1.38±0.03^{NS} (NS)	28.80±6.15** (##)	52.49	12±1.33^{NS} (##)	79.31
F+M	11.80±1.00** (##)	72.31	1.37±0.03^{NS} (NS)	11.20±2.58* (###)	73.91	10±0.98^{NS} (##)	81.25
As+F+M	14.20±2.34* (###)	76.00	1.36±0.04^{NS} (NS)	26.00±4.60** (##)	63.82	12±0.75* (###)	84.21

As: arsenic; F: fluoride; M: melatonin

All the groups were compared with the negative control group using students t-test. Similarly the antioxidant groups were compared with the respective pro-oxidant groups indicated by the symbols in parenthesis.

Significance levels were indicated by *p<0.05, ** p<0.01, *** p<0.001, NS = not significant in comparison to control.
# p<0.05, ## p<0.01, ### p<0.001 , NS= not significant in comparison to respective pro oxidant group, indicated in parenthesis.

Numerical aberrations

A significant rise in hypoploidy was observed in groups exposed to As, F and the combination. Addition of melatonin to the prooxidants led to a remarkable decrease in the number of hypoploids in comparison to the respective prooxidant groups. When compared to control, induction of number of hypoploids was comparable in groups consisting melatonin with As or F separately while melatonin added to the combination demonstrated marked increase in the frequency of hypoploids. The range of percentage amelioration calculated for induction of numerical aberrations was from 79% to 84% (Table II).

CBMN and CBPI

As and F both individually as well as in combination led to significant increase in the frequency of MN induction, in comparison to control values. Groups wherein melatonin was co cultured with the prooxidants demonstrated marked decrease in induction of MN, as compared to respective prooxidant groups. However these antioxidant groups when compared with the control group elucidated remarkable rise in the number of MN. Since there was significant difference between the values of prooxidant and anti oxidant groups thus the percentage amelioration was calculated for these antioxidant groups and the values obtained ranged from 72% to 78% (Table II).

Comet tail length

A noteworthy rise in tail length was observed in the groups consisting both As and F, alone and in combination. Co culturing melatonin with As and F brought about a striking decrease in the tail length leading to values analogous to control (Table III).

Table III: Human peripheral blood cultural was used to perform Comet assay, in duplicates, to analyze the comet tail length in blood cultures after 24 hr *in vitro* exposure to different treatments, as mentioned in the table.

Groups	Control	M	EMS	As	F	As+F	As+M	F+M	As+F+M
Mean ± SE	4.81±0.91	3.16±1.70 NS	4.81±2.78**	11.67±1.54 **	10.90±1.79 *	11.88±1.83 **	5.08±0.92 NS (##)	3.49±0.22 NS (##)	2.19±0.81 NS (###)

As: arsenic; F: fluoride; M: melatonin
All the groups were compared with the negative control group using students t-test. Similarly the antioxidant groups were compared with the respective pro-oxidant groups indicated by the symbols in parenthesis.
Significance levels were indicated by *p<0.05, ** p<0.01, *** p<0.001, NS = not significant in comparison to control.
# p<0.05, ## p<0.01, ### p<0.001 , NS= not significant in comparison to respective pro oxidant group, indicated in parenthesis.

In the present study EMS exhibited genotoxic effect on blood cultures, indicated by significant increase in number of hypoploids, CAs, CBMN and comet tail length values along with marked reduction in CBPI, while the values for melatonin group were comparable to untreated control (Table II, III).

DISCUSSION

The present study evidenced that As as well as F, led to DNA damage to human lymphocyte at the given dose, *in vitro*. The mechanism of action of these prooxidants is not yet established but certain hypotheses have been proposed suggesting the role of ROS. Being a prooxidant, As causes glutathione depletion, protein enzyme oxidation, lipid peroxidation along with DNA oxidation and adducts formation.[22] In addition hemeoxigenase and peroxidase are induced by As in various human cell lines.[23] Huang and Lee[24] demonstrated that As treatment altered the morphology and the function of mitotic apparatus, which implies that As treatment may slow down the rate of depolymerisation of mitotic spindle. Since aneuploidy and micronuclei can be the result of the failure of spindle assembly check points, this can explain the frequency of MN and hypoploidy observed in the present study.

Metabolic methylation of inorganic As to dimethyl arsenous acid is involved in induction of DNA damage and DNA single strand breaks resulting from inhibition of repair polymerization.[25] In addition As mediated DNA protein interaction could induce protein associated DNA strand breaks and may provide an explanation for the observed chromosomal aberrations. Our results are concordant with these studies presenting a significant increase in the frequency of CBMN and chromosomal anomalies. Guillamet et al[26] have also demonstrated *in vitro* DNA damage caused by As using comet assay in human lymphoblastoid cell line, in support of our data.

As far as F is concerned there are many groups that don't agree with the genotoxic potential of F. Ling and Chen[27] concluded from their study that F induces oxidative stress, DNA damage and cell cycle changes in rat oral mucosal cells and hepatocytes, similar to our observation in lymphocytes. Nair et al[28] reported induction of SCE and Albanese[29] demonstrated induction of CAs in cultured human lymphocytes exposed to F, indicating DNA damage as observed in our results. F has also been found to be clastogenic *in vitro*, because of effect on enzymes involved in DNA synthesis or repair rather, providing probable justification for the reported F induced CAs in the present study.[30] In agreement to our results Meng and Zhang[31] also observed a significant increase in frequency of CAs and MN in workers of phosphate fertilizer factory, exposed to fluoride in the form of SiF_4 and HF.

Melatonin as an antioxidant can exert its effect either directly via free radical scavenging or indirectly via membrane receptors by stimulating the antioxidant enzymes.[15] Vijaylaxmi et al.[32] exemplified the protection of human lymphocytes from genetic damage induced by gamma radiation, observed as the reduction in frequency of radiation induced CAs after melatonin pretreatment. Thus suggesting mitigative action of melatonin against oxidative stress, corroborating our results. Bard et al[33], reported that the frequency of micronuclei in polychromatic erythrocytes in blood and of chromosomal aberrations in sperm were significantly reduced in melatonin pretreated mice compared to irradiated mice. Consequently suggesting the protective effect as concluded in the present study. Similar results were reported in a study by Vijayalaxmi et al[34,35] demonstrating a decrease in production of micronuclei after whole body irradiation in mice, as well as reduced the comet tail in irradiated human blood cells after melatonin pretreatment thus presenting the radioprotective effect of melatonin. All these studies summarize that melatonin could efficiently reduce the genotoxic indices, in corroboration to this report

Production of ROS is considered as the mechanism of radiation induced genotoxicity and the ability of melatonyl cation radical to scavenge toxicity of singlet oxygen gives way to reduction in formation of highly reactive hydroxyl radicals and peroxinitrite anions. Melatonin can thus inhibit DNA damage induced by ROS because of its antioxidant activity, as reported in the present study.

In conclusion, melatonin remarkably reduced frequency of chromosomal aberrations and micronuclei induced *in vitro*, by As and/or F, in human blood cultures and this ameliorative action could be attributed to its powerful antioxidant activity. Consequently, melatonin could be further studied in search of an antidote to fight against the global water contaminants As and F. Therefore the results of present study posses a striking testimony on mitigation of As and/or F by melatonin.

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