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Genotoxicity of Synthetic Food Colorants

V. R. Swaroop, D. Dinesh Roy and T. Vijayakumar

School of Environmental Sciences, Mahatma Gandhi University, Kottayam, Kerala 686560, India

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Abstract: A study was conducted to evaluate the genotoxicity, if any, of the permitted synthetic food colorants used in India. Eight synthetic food colorants namely Erythrosine (E127), Tartrazine (E102), Ponceau 4R (E124), Sunset Yellow FCF (E110), Brilliant Blue FCF (E133), Fast Green FCF (E143), Carmoisine (E122) and Indigo Carmine (E132) and their combination are used in sweets namely Ladu, Jilebi and Halwa in Calicut and suburban areas of Kerala, in India. The genotoxicity of the colorants alone and in combinations at different concentrations were evaluated by Cytokinesis Block Micronucleus (CBMN) Assay. It was observed that all the above colorants and their combinations could cause genotoxicity to human lymphocytes even at the permissible concentration of 100 ppm as per PFA (Prevention of Food Adulteration) Act of India. The toxicity varied from dye to dye and was proportional to their concentration. Combination of colors showed more toxicity than the individual components. Toxicity could be reduced drastically by reducing the concentration of the dyes at least 50% below the permissible limit. Permitted synthetic food colorants even at the permissible limit should be used with caution. This study demonstrated the need for redefining the permissible limit of the food colorants based on Admissible Daily Intake (ADI) as being practiced in developed countries.

Key words: Synthetic food colorants, genotoxicity, CBMN (Cytokinesis Block Micronucleus) Assay, somatic DNA damage, food adulteration, ADI (Admissible Daily Intake).

1. Introduction

The Codex Alimentarious Commission (CAC) has defined "Food Additive" as any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment , packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly) in it or its by products becoming a component or otherwise affecting the characteristics of such foods [1]. Among the synthetic food additives, food colorants are of major concern in India. Studies have shown that the intake of non-permitted toxic colors like rhodamine, auramine and orange II poses severe health hazards [2, 3]. Even permitted colors are being used in excess of the statutory limit or in foods in which they are not permitted [4-8].

Permitted colors are also found to have adverse effects. The adverse reactions vary from urticaria to dermatitis, angioedema and exacerbation of the asthmatic patients [9]. Natural and synthetic dyes, especially those used in food stuffs have become potential suspects for causing cancer [10, 11]. For the majority of the food additives JECFA/FAO has assigned "Admissible Daily Intake Dose"-ADI, which are often temporary and emphasized the need for further genotoxic evaluation, since a number of them are reported to be genotoxic below the ADI dose. Some food colors and their side effects have been investigated in animal studies and it has been found that their mutagenicity varies widely, depending on the dose consumed [11]. The ADI of permitted colors varies from 0.1 mg kg^{-1} body weight for erythrosine to 25 mg kg^{-1} body weight for fast green FCF. It is important to monitor the total daily intake of all food colors since the ADI level of any colors should not be

Corresponding author: V. R. Swaroop, M.Sc., research field: food additives. E-mail: vr_swaroop@yahoo.co.uk.

exceeded [9]. Unfortunately in India no such studies are available and a maximum concentration of 100 ppm was fixed arbitrarily for all colors except in canned foods, jams and jellies by PFA Act (Prevention of Food Adulteration Act, India, 1954). In an earlier study conducted by us on the three major sweets namely Halwa, Ladu and Jilebi showed color concentration two to four times above the permissible limit [12].

DNA electrophoretic mobility experiments showed that colorants are capable of strong binding to linear dsDNA causing its degradation. Studies have shown that most of these colorants bind directly to the DNA and cause both structural and numerical anomalies [13, 14]. Many methodologies have been developed over the past 20 years, varying from cytogenetic techniques that can give a broad assessment of mutagenic events to adduct assays that are designed to detect exposure to specific agents. No single technique can satisfy all the requirements, and it is now realized that a variety of methods can be combined to provide an effective screening system [15, 16].

The most important method used to determine the chromosome damage caused by chemicals is by CBMN (Cytokinesis Block Micronucleus) assay. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. In its current basic form the CBMN assay can provide, the following measures of genotoxicity and cytotoxicity: chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition and necrosis and apoptosis by simple morphological criteria [17].

The first attempt to assess the risk of food colorants was made by Rao and Sudershan [18]. An attempt was made by us to estimate the concentration of the various colorants in sweet items in India. But studies on the effects of colorants on DNA are scanty and inconclusive. Moreover no attempts were made in India to assess the genotoxiciy of these colorants on humans in the permitted dose of 100 ppm. Hence the present study was undertaken to evaluate the DNA damage, if any, induced by the colorants at various doses. An attempt is also being made to see whether the genotoxicity can be reduced by reducing the concentration of the colorants below the permissible limit.

2. Materials and Methods

One thousand samples of the most popular sweets (Ladu, Jilebi and Halwa) were collected at random from different parts of Calicut and suburban area of Kerala (India). The colorants used in the food items were isolated, characterized by chromatographic technique and quantified spectrophotometrically.

2.1 Isolation and Characterization of Colorants

The samples were defatted with three changes of petroleum ether and treated with 50 mL of 2% ammonia in 70% ethanol. Wool dyeing technique was employed to extract the colorants from the above solution. Under acidic condition the wool took any synthetic dye if present. The colorants from the dyed wool were stripped by making the solution alkaline with ammonium hydroxide solution. The extracted colors were identified by ascending paper chromatography using the Butanol: Water: Acetic acid (20:15:5) solvent system.

2.2 Quantification of Colorants

The quantification of various colorants was undertaken by measuring absorbance at respective λ_{max} along with standards employing a spectrophotometer (Shimadzu UV-2450) [19].

2.3 Cytokinesis Block Micronucleus (CBMN) Assay

The genotoxicity of the colorants alone and in combinations at different concentrations were evaluated by Cytokinesis Block Micronucleus (CBMN) Assay. Eight synthetic coloring agents namely Erythrosine (E127), Tartrazine (E102), Ponceau 4R (E124), Sunset Yellow (E110), Brilliant Blue FCF

(E133), Fast Green (E143), Carmoisine (E122), and Indigo Carmine (E132) and their combinations were detected at varying concentrations in these food items. Samples of the above colorants were purchased from M/s. Sigma chemicals. These colors and their four different combinations namely Sunset Yellow + Tartrazine, Sunset Yellow + Carmoisine + Brilliant Blue, Brilliant Blue + Tartrazine, Brilliant Blue + Tartrazine + Carmosine were dissolved in water and sterilized by membrane filtration (Pore size 0.2 micron). Blood samples from healthy young adult volunteer without any history of chronic illness, drug intake and or any life style associated bad habits were collected with informed consent. Lymphocytes were separated from these samples using Lymphoprep (M/s. Pharmacia). Four parallel Lymphocyte microcultures in duplicate were set up for each sample to evaluate the extent of genotoxicity produced by various food coloring agents as described by Fenech [20]. Culture A was kept as control and coloring agents and their above combinations were added to Culture B, C and D with varying concentrations of 100, 200 and 500 μg mL^{-1}, respectively at the time of culture initiation. Cytochalasin B (Sigma) at a concentration of 4.5 μg mL^{-1} was added to all the 4 cultures at 44th hour to block the cell division at Cytokinesis. The cultures were harvested at 72nd hour; slides were prepared and stained with 10% Geimsa stain. Observed under a plan achromatic microscope for enumerating the frequency of micronuclei among 1000 binucleated cells and recorded. The criteria for enumeration were: (1) cells should have two nuclei of approximately equal size, (2) the 2 nuclei may be attached by a fine nucleoplasmic bridge, (3) the two nuclei may overlap slightly or touch each other at the edges and (4) cells should not contain more than 6 micronuclei.

The data were enumerated and multivariate analysis was performed using Statistical Package for Social Sciences (SPSS).

3. Results and Discussion

The percentage distribution of different colors and their concentration in the sweets studied is given in Table 1. Seventy five percentage of the samples contains colorants below the permissible limit of 100 ppm. But 13.6% of the samples had color concentration up to 200 ppm and the remaining 11.4% had color concentration above 200 ppm i.e. more than double the permissible limit.

The mean CBMN frequency with different colors and at different concentrations is given in Table 2. The mean CBMN frequency of the control samples (culture A1 and A2) was 10 ± 0. The cultures B1 and B2 showed a mean CBMN frequency of 12 ± 0.7. The cultures C1 and C2 showed a mean CBMN frequency of 12.8 ± 0.8 and the cultures D1 and D2 showed a mean CBMN frequency of 13.7 ± 0.7. The multivariate analysis revealed that these differences had statistical significance (F = 25.824; P = 0.001). Pair wise comparison for different concentrations were also showed statistically significant differences (Tables 3 and 4).

But in the case of color combinations the genotoxicity was comparatively higher when the total concentration of the combination was the same as that of the individual colorants. The CBMN frequency was reduced drastically by reducing the concentration of

Table 1 Distribution of samples according to concentration of colorants.

Item	Color	Concentration of Colorants		
		< 100 ppm	100-200 ppm	> 200 ppm
Laddu	Yellow (n=100)	73	18	9
n=300	Red (n=100)	85	12	3
	Orange (n=100)	72	13	15
Jilebi	Yellow (n=100)	71	19	10
n=300	Red (n=100)	76	16	8
	Orange (n=100)	78	10	12
Halwa	Yellow (n=80)	70	7	3
n=400	Red (n=80)	57	14	9
	Orange (n=80)	65	6	9
	Brown (n=80)	51	11	18
	Green (n=80)	52	10	18

The figures in parenthesis denote the number of samples. The chemicals used for the colors are: 1. Yellow-Tartrazine, 2. Red-Sunset Yellow, 3. Orange-Tartrazine + Sunset yellow, 4. Brown-Tartrazine + Carmoisine + Brilliant Blue or Sunset Yellow + Carmoisine + Brilliant Blue, 5. Green-Tartrazine + Brilliant Blue.

Table 2 Frequency of micronucleus induced by food colors according to various concentrations.

Food Color	Culture A	Culture B	Culture C	Culture D
	Control	100 μg mL^{-1}	200 μg mL^{-1}	500 μg mL^{-1}
Erythrosine	10	11	11.6	13.2
Tartrazine	10	10.6	12.2	13
Ponceau 4R	10	12.1	12.5	14
Sunset Yellow	10	11.4	12.2	13
Brilliant Blue	10	11.8	12	12.4
Fast Green	10	11.6	12.5	13.6
Carmoisine	10	12.6	13.4	14.2
Indigo Carmine	10	12.8	13.8	14
Sunset Yellow + Tartrazine	10	12	12.8	14
Sunset Yellow + Carmoisine + Brilliant Blue	10	13	13.4	14.4
Brilliant Blue + Tartrazine	10	12.4	13	13.2
Brilliant Blue + Tartrazine + Carmoisine	10	12.6	14.3	15
Mean	10	12 ± 0.7	12.8 ± 0.8	13.7 ± 0.7

Table 3 Multivariate analysis.

Multivariate Tests	Value	*F*	Hypothesis df	Error df	*P*
Pillai's trace	0.906	25.824	3	8	0.001

Table 4 Pairwise comparison.

		Pairwise comparisons	
(I) Factor1	(J) Factor1	Mean difference (I-J)	
Culture A	Culture B	-0.9	0.016
Culture A	Culture C	-1.9	0.002
Culture A	Culture D	-2.8	0.000
Culture B	Culture C	-1.0	0.002
Culture B	Culture D	-1.9	0.000
Culture C	Culture D	-0.9	0.002

the colorants to 50 ppm i.e, 50% of the present permissible limit of 100 ppm as per the PFA Act of India. Comparison of the genotoxicity of all the food colorants and their combinations at 100 and 50 ppm are given in Table 5.

Changing life styles and use of packaged food items has influenced the food consumption pattern of the average Indians [21]. In India majority of food manufacturers are running small scale and cottage industries and hence they are unable to restrict the use of colorants or any other additives as per stipulations of the PFA due to ignorance and/or negligence. This finding is well in agreement with the earlier reports that additives are not used as per the regulations under PFA Act [18].

The modern technological advancement in food industry has resulted in the use of variety of food colors alone or in combination [22]. Studies have shown that the food colors might cause health hazards affecting kidneys and causing allergies and gastrointestinal cancer. Food colorants were reported to be toxic to human lymphocytes *in vitro* and it seems that they bind directly to DNA [14]. The present study clearly demonstrates a statistically significant increase in the micronuclei frequency induced by various food colors. The food colorants induced mitotic division abnormalities in varying concentrations below and above the permissible limit.

Das and Mukherjee [10] had shown that food coloring agents, amaranth, erythrosine and tartrazine at the concentration of 8 mM caused high genotoxicity, cytostaticity and cytotoxicity. The frequency of SCEs/cell was increased 1.7 times over the control level. Furthermore, spectroscopic titration studies for the interaction of these food additives with DNA showed that these dyes bind to calf thymus DNA and distinct isosbestic points are observed clearly suggesting binding of the dyes to DNA. Additionally DNA electrophoretic mobility experiments showed that these colorants are obviously capable for strong binding to linear dsDNA causing its degradation. Evaluation of the data and curves were obtained after

Table 5 Comparison of the frequency of micronucleus induced by food color at 100 μg mL^{-1} (permissible limit) and at 50 μg mL^{-1} (half the permissible limit).

Food Color	Culture A	Culture B	Culture E
	Control	100 μg mL^{-1}	50 μg mL^{-1}
Erythrosine	10	11	10.7
Tartrazine	10	10.6	10.6
Ponceau 4R	10	12.1	11.2
Sunset Yellow	10	11.4	10.8
Brilliant Blue	10	11.8	10.7
Fast Green	10	11.6	10.6
Carmosine	10	12.6	10.8
Indigo Carmine	10	12.8	11
Sunset Yellow + Tartrazine	10	12	11
Sunset Yellow + Carmosine+ Brilliant Blue	10	13	11.1
Brilliant Blue + Tartrazine	10	12.4	10.8
Brilliant Blue+ Tartrazine + Carmosine	10	12.6	10.6

quantitative and qualitative analysis of the lines of the gel by an analyzer computer program [11]. In this study it was observed that almost all the food colorants were toxic even at the permissible limit of 100 ppm.

The ADI for permitted synthetic colors varies from 0.1 to 25 mg kg^{-1} bodyweight, i.e. a difference of 250 times in developed countries [1]. The rationale of fixing a uniform regulatory limit of 100 ppm to the permitted colors in all foods by the PFA is not justified. There is a need to develop regulatory limits for additives based on risk assessment taking into consideration of food habits and technological necessity. A study was attempted to evaluate the genotoxic effect of erythrosine [23] on Swiss albino mice. Calculation of mitotic index and scoring of chromosomal aberrations were carried out. The dose-dependent decrease in cell proliferation observed was significant. In the present study a significant increase in the CBMN frequency with increased dose of erythrosine exposure was observed which is in agreement with the previous report.

Chromosomal aberrations induced by Ponceau 4R (an azo food dye) and beta-carotene (a natural food color) were studied on bone marrow cells of mice *in vivo*. The results indicated that Ponceau 4R was more clastogenic than beta-carotene. Ponceau 4R was found to have a minimum effective dose of 4 mg which induced a significant number of chromosome aberrations. This dose is also the recommended as "admissible daily intake". In so far as genotoxicity is concerned Ponceau 4R should be delisted as a food dye [24]. Indigo carmine was also reported to cause potential damage to genetic material *in vitro* [25, 26]. The present evaluation of genotoxicity of ponceau 4R and indigo carmine also showed that these two dyes are genotoxic which is well in agreement with the above findings.

Sunset Yellow has been shown to cause allergic or intolerance reactions in certain people, particularly those with a pre-existing sensitivity to aspirin, but no mutagenic effect was reported [27]. In the present study no attempts were made to study the allergic response of this colorant but the genotoxicity observed was comparatively lower than many of the other colorants as well as color combinations.

Erythrosine was reported to cause chromosomal structural aberrations which may be suggestive of its mutagenicity [13]. Low dose of erythrosine was reported to produce few adverse effects in reproductive and neurobehavioral parameters in mice [28]. Erythrosine did not induce DNA repair in rat hepatocytes *in vitro* at concentrations up to 1 mM, or *in*

vivo after an oral dose of 200 mg kg^{-1} body weight [29]. In the present study the genotoxicity observed with Erythrosine was lower than all colorants except tartrazine.

4. Conclusion

The observations indicate that permitted synthetic food colors can induce genotoxicity in humans even at the permissible limit. The CBMN frequency was found to be directly proportional to the concentration of the colorants. Combinations of colors are found to be more toxic compared to the same concentration of individual colors. The toxicity could be reduced by reducing the concentration of the colorants below the permissible limit. This study clearly indicates the need for redefining the maximum permissible limit for food colorants. Instead of arbitrarily fixing the limit at 100 ppm, each dye should have individual limit based on well controlled genetic studies.

References

[1] CAC, Discussion Paper on the Use of Colors in Foods, Joint FAO/WHO Food Standards Programme, Codex Committee on Food Additives and Contaminants, Codex Alimentarius Commission, CX/FAC 00/9, 1999.

[2] S.K. Khanna, L.P. Srivastava, G.B. Singh, Comparative usage pattern of synthetic dyes in yellow to orange colored eatables among city and rural markets, Indian Food Packer. 40 (1986) 33-37.

[3] P. Mathur, Risk analysis of food adulterants, Ph.D. Thesis, National Institute of Nutrition, Hyderabad, India, 2000, pp. 146-183.

[4] R.V. Bhat, P. Mathur, Changing scenario of food colors in India, Current Science 74 (1998) 198-202.

[5] R. Padmaja, P.R. Jonnalagadda, R.V. Bhat, N.A. Naidu, Type and extent of colors used in ready-to-eat (RTE) foods prepared in the non-industrial sector, Int. J. Food Science and Technology 39 (2004) 125-131.

[6] R. Pratima, R.V. Bhat, A comparative study on the synthetic food colors usage in foods procured from urban and rural areas of Hyderabad, Nutrition and Food Science 33 (2003) 230-235.

[7] R. Pratima, V.B. Ramesh, R.V. Sudershan, P.T. Krishna, N.A. Naidu, Exposure assessment of synthetic food colors in a selected population in Hyderabad, India, Food Additives and Contaminants 21 (2004) 415-421.

[8] R. Pratima, V.B. Ramesh, R.V. Sudershan, P.T. Krishna, N.A. Naidu, Consumption of synthetic food colors during festivals in Hyderabad, India, British Food Journal 105 (2005) 276-284.

[9] WHO, Toxicological Evaluation of Certain Food Additives and Contaminants, WHO Food Additives Series No. 28, World Health Organization, Geneva, 1991.

[10] A. Das, A. Mukherjee, Genotoxicity testing of the food colors amaranth and tartrazine, Int. J. Hum. Genet. 4 (2004) 277-280.

[11] S. Tsuda, M. Murakami, N. Matsusaka, K. Kano, K. Taniguchi, Y.F. Sasaki, DNA damage induced by red food dyes orally administered to pregnant and male mice, Toxicol Sci. 61 (2001) 92-99.

[12] V.R. Swaroop, D. Dinesh Roy, T. Vijayakumar, Identification and estimation of coloring agents in sweets (Jilebi, Laddu, and Halwa) of kozhikode, malabar, kerala, Beverage and Food World (2011). (in press)

[13] A. Hamdy, A.A. Mekkawy, A. Massoud, M. El-zawahry, Mutagenic effects of the food color erythrosine in rats, Problems of Forensic Sciences XLIII (2000) 184-191.

[14] P. Mpountoukas, A. Pantazaki, E. Kostareli, et al., Cytogenetic evaluation and DNA interaction studies of the food colorants amaranth, erythrosine and tartrazine, Food Chem Toxicol. 25 (2010) 37-43.

[15] M.L. Mendelsohn, An introduction to biological dosimetry, Prog. Clin. Biol. Res. 372 (1991) 1-10.

[16] C.C. Harris, Molecular epidemiology: overview of biochemical and molecular basis, in: J.D. Groopman, P.L. Skipper (Eds.), Molecular Dosimetry and Human Cancer, CRC Press, Boca Raton FL, 1991, pp. 15-26.

[17] M. Fenech, Mathematical model of the *in vitro* micronucleus assay predicts false negative results if micronuclei are not scored specifically in binucleated cells or cells that have completed one nuclear division, Mutagenesis 15 (2000) 329-336.

[18] P. Rao, R.V. Sudershan, Risk assessment of synthetic food colors: a case study in Hyderabad, India, Int. J. Food Safety, Nutri Pub Health 1 (2008) 68-87.

[19] S. Ranganna, Analysis and Quality Control for Fruits and Vegetable Products, 2nd ed., Tata McGraw-Hill Publishing Company Limited, New Delhi, 2007, pp. 286-288.

[20] M. Fenech, The *in vitro* micronucleus technique, Mutat Res. 455 (2000) 81-95.

[21] B. Sridevi, U.M. Reddy, Consumption of processed food products among urban families, Indian Food Industry 17 (1998) 290-295.

[22] K.F. Kiple, K.C. Ornelas, Contemporary Food Related Issues, II United Kingdom, The Cambridge University Press UK, 2000, pp. 1667-1676.

[23] J. Vivekanandhi, C.P. Anitha Devi, K. Jayaraman, L.

Raghavan, Effect of erythrosine on testicular function of mice, Toxicol. Int. 13 (2006) 119-125.

[24] K. Agarwal, A. Mukherjee, A. Sharma, *In vivo* cytogenetic studies on male mice exposed to Ponceau 4R and beta-carotene, Cytobios 74 (1993) 23-28.

[25] S. Abbruzzetti, C. Viappiani, D.H. Murgida, et al., Non-toxic water-soluble photocalorimetric reference compounds for UV and visible excitation, Chem. Phys. Lett. 304 (1999) 167-172.

[26] Y. Rhee, J. Termini, M. Valentine, Oxidative base damage in DNA detected by reverse transcriptase, Nucl. Acids. Res. 23 (1995) 3275-3282.

[27] Health Canada, Food Additive Dictionary, 2007, available online at: http://www.hc-sc.gc.ca/fn-an/securit/addit/diction/index_e.html.

[28] T. Tanaka, Reproductive and neurobehavioral toxicity study of erythrosine (Red 3) administered to mice in the diet, Food Chem Toxicol. 39 (2001) 447-454.

[29] D. Kornbrust, T. Barfknecht, Testing of 24 food, drug, cosmetic, and fabric dyes in the *in vitro* and the *in vivo*/*in vitro* rat hepatocyte primary culture/DNA repair assays, Environ. Mutat. 7 (1985) 101-120.