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Mutagenicity Evaluation of an Azo Dye - Reactive Red Using the Ames Test and in Vitro Cytokinesis Block Micronucleus (CKB-MN) Assay

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ABSTRACT
Azo dyes are extensively used in the textile industry and in printing inks, plastics and other materials. There are numerous studies that have implicated the adverse health effects that azo dyes may pose and it has been clarified that the biological activity of these azo dyes depends on their ability to generate reactive metabolites. The present study aimed to evaluate the genotoxic potential of one such azo dye, Reactive red using the Ames test and the Cytokinesis - Block micronucleus (CKBMN) assay in human lymphocytes. In the Ames test, no mutagenic response was seen in any of the test concentrations and tester strains, generally, evidenced as a two-three fold increase over the concurrent solvent controls. In the CKBMNT, the percentage micronucleatedbinucleate cells in the solvent control and in the treated groups, (4-hour, +/-S9 and continuous, -S9), fell within the historical range of the laboratory. Considering the lack of a 2-3 fold increase in the number of revertant colonies in reactive red-treated plates with respect to the solvent controls, it may be concluded that the test substance is non-mutagenic in *Salmonella typhimurium* tester strains, at the doses tested and the experimental conditions employed.The percentage micronucleated binucleate cells of all Dye- treated cultures fell within or close to the historical control values and were not significantly different from the concurrent solvent control cultures. No dose-dependent increase in the frequency of micronuclei was recorded. Thus, Reactive red was considered non-mutagenic in the Ames test and non genotoxic in the *in vitro* micronucleus assay in human peripheral blood lymphocytes at the doses tested and the experimental conditions employed.

Key words : Reactive red, ames test, micronuclei, lymphocytes

INTRODUCTION

Azo dyes are extensively used in the textile industry and in printing inks, plastics and other materials. Reductive cleavage and generation of aromatic amines upon contact with biological secretions such as sweat, saliva and gastric juices make azo dyes harmful to humans (Pielesz et al., 1999, 2002);their metabolic intermediates have been known to induce genotoxicity and carcinogenicity (Khehra et al., 2006). The mutagenic/ carcinogenic nature of these dyes is presumed to be due to the direct action of the parent molecules or through mechanisms such as free radical generation and or formation of aryl amines during biotransformation (Chung et al., 1992; Collier et al., 1993; Rajaguru et al., 1999).

There are numerous studies that have implicated the adverse health effects that azo dyes may pose and it has been clarified that the biological activity of these azo dyes depends on their ability to generate reactive metabolites (Brantom, 2005). The present study aimed to evaluate the genotoxic potential of one such azo dye, **Reactive red** using the Ames test in Salmonella typhimurium and the Cytokinesis - Block micronucleus (CKBMN) assay in human lymphocytes.

Material and Methods

Chemicals and reagents

Reactive red dye was obtained from Sigma, USA. The dye was soluble in water.

The Ames Salmonella reverse mutation test and the *in vitro* micronucleus test were conducted.The Ames test was performed as per Maron and Ames (1983). A test substance was considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA98, TA100 and TA102) or thrice (strains TA1535 and TA1537) the colony counts of the corresponding solvent control was observed. The number of revertant colonies per plate, its frequency, the statistical significance of any increase in any of the strain and its correlation to the test concentration in a given time period, was considered for evaluating the mutagenic potential of the substance.

The *in vitro* micronucleus assay was performed using human peripheral blood lymphocytes as recommended by Fenech (2000). The scheme of treatment using in the present study is as follows

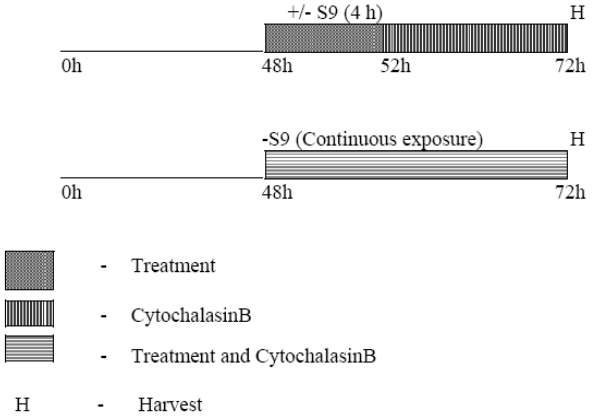

Figure 1: Scheme of Treatment

Cultures were treated with the test material\solvent\positive controls in the presence and absence of S9, at 48-hour from initiation of the cultures. Cells were treated in the presence of the test material for 4-hour in the presence and absence of metabolic activation system (S9), and continuously for 24-hour in the absence of metabolic activation system (S9). For 4-hour exposure with S9 the medium contained 0.25 ml of S9 mix. After the treatment period, cells were washed off the test material and cultured in freshly prepared medium with Cytochalasin-B and all additions (except PHA-M and S9). Cultures were incubated in a humidified 5% CO_2 atmosphere at 37 ± 0.5°C for 1.5 cell cycle (~36 hrs). For continuous exposure in the absence of S9, cultures were treated similarly as detailed above except that the cultures were incubated continuously in the presence of the test substance/solvent/ positive controls and cytochalasin-B for 1.5 cell cycle times. The pH of all treatment conditions and signs of contamination were recorded. Duplicate cultures were maintained for each treatment.

Based on a preliminary range-finding test (data not shown), the following concentrations were tested:

Exposure time	Concentration
4 hour with S9	5000, 2500 & 1250 µg/mL
4 hour without S9	5000, 2500 & 1250 µg/mL
Continuous without S9	5000, 2500 & 1250 µg/mL

At the end of the treatment period, cultures were terminated and after hypotonic shock (0.075MKCl) and fixation of the cells, smears of the cell suspension were made on glass slide using a Cytospin (Shandon Cytospin4, USA). Cell preparations were stained with 3% Giemsa (Merck, UK).

Analysis

Cytotoxicity, in this case, cytostasis was assessed by calculating the Cytokinesis-Block Proliferation Index (CBPI). About 500 cells (per culture) with one, two and multiple nuclei were classified for CBPI analysis. For ascertaining micronuclei frequencies, 2000 bi-nucleated cells per concentration of test material, solvent/negative and positive controls, equally divided amongst the duplicates were analyzed.

Results

Ames test

The data on the revertant colonies per plate and average histidine revertant colonies for the various test concentrations and the concurrent solvent and positive controls with and without metabolic activation is presented in **Tables 1 and 2.**

No marked or consistent differences in the number of revertant colonies were observed in the test concentrations and concurrent solvent controls in all the tester strains with (10% S9) and without exogenous mammalian S9 activation system.

The genotypes of all the tester strains were assessed to be intact. In the precipitation test, no visible turbid precipitate was obtained in the top agar mix even at the highest concentration employed (5000 µg/plate) and this dose was used as the top dose for the range finding study.

A **range finding study - pre-incubation method** was performed with two tester strains, TA 98 and TA 100, employing seven test concentrations - 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate. Simultaneously, cultures of controls received sterile millipore water. No signs of cytotoxicity were observed in any of the test concentrations with and without S9. No mutagenic response was seen in any of the test concentrations and tester strains, generally, evidenced as a two-three fold increase over the concurrent solvent controls.

Based on the negative results obtained from the **range finding study**, a confirmatory assay was performed with the **main study - pre-incubation method** with all the five tester strains (TA 98, TA 100, TA 102, TA 1535, and TA 1537). Test concentrations employed were 312.5, 625, 1250, 2500 and 5000 µg/plate with (10% S9) and without S9. Concurrent solvent controls and strain-specific positive controls were maintained.

In the main study, no contamination was observed in any of the treatment conditions and methods. The number of revertants recorded in the treated plates was well within the range of the historical control data. The strain-specific positive controls demonstrated positive response in the respective tester strains. The assay thus met the criteria for a valid test. There was no sign of cytotoxicity in any of the test concentrations with and without S9. No mutagenic response was seen in any of the test concentrations and tester strains, generally, evidenced as a two-three fold increase over the concurrent solvent controls.

Considering the lack of a 2-3 fold increase in the number of revertant colonies in Reactive red-treated plates with respect to the solvent controls, it may be concluded that the test substance is non-mutagenic in *Salmonella typhimurium* tester strains, at the doses tested and the experimental conditions employed.

CKB-MN assay

Reactive red dye was evaluated for its genotoxic potential in cultured human peripheral blood lymphocytes. **Table 3** summarizes the results obtained. The percentage micronucleated binucleate cells in the solvent control cultures fell within the historical control of the laboratory. The positive control chemicals, Mitomycin C, Colchicine and Cyclophosphamide induced a significant increase in percentage micronucleated binucleate cells compared to concurrent solvent control cultures. The assay was therefore considered valid. In all three treatment regimes (4-hour, +/-S9 and continuous, -S9), treatment of cultures with Reactive red dye, resulted in an acceptable dose related inhibition in % Cytostasis. The percentage micronucleated binucleate cells of all Dye-treated cultures fell within or close to the historical control values and were not significantly different from the concurrent solvent control cultures. No dose-dependent increase in the frequency of micronuclei was recorded. Thus, Reactive red dye was considered non genotoxic in the *in vitro* micronucleus assay in human peripheral blood lymphocytes at the doses tested and the experimental conditions employed.

Discussion

Many dyes are existing chemicals and have very few data on toxicity and other health effects (Brown and De Vito, 1993, Walters et al., 2005, Schneider, 2004). Hitherto, there is not much literature on the genotoxicity of Reactive dyes; hence the present study was initiated. The ames test and the *in vitro* cytokinesis block micronucleus (CKB-MN) assay was used to study the genotoxic potential of this dye. The assay is based on the inhibition of actins by cytochalasin-B at the cytokinesis stage of cell division and identifies chemicals (or their metabolites) that inhibit the cell cycle and induce micronuclei (Fenech and Morley, 1985). Considering the ease of performance and interpretation, this assay was employed.

In the current investigation, two Reactive red dyes were evaluated using the Ames test and the *in vitro* micronucleus assay both in the presence and absence of an exogenous metabolic activation system (S9).

There was no increase in the frequency of the spontaneous revertants in any of the concentrations tested in the five tester strains, thus leading to the conclusion that the dye tested is non-mutagenic in the Ames test.

There was no dose-dependent increase in the frequency of micronucleus induction. Few studies have demonstrated the *in vitro* mutagenicity of azo dyes using the micronucleus assay. Harrington - Brock et al (1991) reported that solvent red 1 an azo dye was mutagenic upon exogenous metabolic activation *in vitro* using the micronucleus assay and mouse lymphoma assay. Tsuboy (2007) reported that the azo dye disperse blue 291 increases micronuclei frequency in a dose dependent manner when tested on human hepatoma cell line (HepG2). Chequer et al. (2009) reported an increase in the frequency of micronuclei in human lymphocytes treated with the azo dyes Disperse Red 1 and Disperse Orange 1 in a dose-dependent manner. However, in the present study, there was no increase in the frequency of micronuclei in human lymphocytes treated with the dyes.

In general, there are three mechanisms by which azo dyes are metabolized (Brown and DeVito 1993):

1. Reductive cleavage of the azo bond and subsequent release of free aromatic amines resulting in mutagenicity.
2. Oxidation of free aromatic amine group belonging to the azo dye structure.
3. Activation of azo dyes through direct oxidation of the azo linkage resulting in the formation of electrophilic diazonium salts.

In all the three mechanisms, reactive metabolites are formed that interact with the DNA/ RNA molecules resulting in mutagenicity.

There are reports that demonstrate the lack of mutagenicity of certain azo dyes as is the case with the current study. These reports are related to the effects of alkyl groups on the mutagenicity of azo-containing compounds. Solvent Yellow 56 (N, N- diethyl -4-phenylazoaniline), *N*,*N*-(di-*n*-propyl)-4-phenylazoaniline) and *N*,*N*-(di-*n*-butyl)-4-phenylazoaniline have all been reported to be non-carcinogenic although they carry an azo group in their structure. Hence the size of the alkyl group plays a major role in determining the mutagenicity of a given azo compound.

There are also reports that demonstrate that the mutagenicity of dyes was reduced or removed with increasing size of the alkoxy substituent (Esancy et al.1990). In one compound, *N*, *N*-dimethyl-4-aminoazobenzene derivatives, when the *N*-methyl groups were replaced by a hydroxyethyl or benzyl group, they were non-carcinogenic, which suggest that if the structure of an azo compound is dominated by bulky alkyl substituent, the formation of mutagenic metabolites is hindered.

With the present results it is proposed that, due to its inherent structure, Reactive red dye could not be metabolized to form any DNA-reactive metabolites even in the presence of an exogenous metabolic activation system (S9) and thus there was no increase the frequency of micronuclei in human peripheral blood lymphocytes. The lack of mutagenicity observed can also be accredited to the same reason. Hence, it can be concluded that, Reactive red dye is non-genotoxic in *the in vitro* micronucleus assay in human peripheral lymphocytes and non-mutagenic as evidenced by the Ames test under the experimental conditions and concentrations employed.

Group		Solvent Control	Concentrations µg/plate					Positive Control
			312.25	625	1250	2500	5000	
TA 100	Plate 1	215	210	210	210	207	209	985
	Plate 2	218	206	208	207	212	207	982
	Plate 3	217	210	209	208	211	210	1047
A.H.R± S.D		216.67 ± 1.53	208.67±2.31	209.00 ± 1.00	208.33 ± 1.53	210.00 ± 2.65	208.67 ± 1.53	1004.67±36.69
TA 102	Plate 1	348	338	334	334	336	330	987
	Plate 2	347	328	327	326	325	327	977
	Plate 3	351	324	341	327	328	325	1008
A.H.R ± S.D		348.67 ± 2.08	330.00±7.21	334.00± 7.00	329.00± 4.36	329.67± 5.69	327.33±2.52	990.67±15.82
TA 1535	Plate 1	44	41	39	37	39	36	243
	Plate 2	43	39	41	39	40	37	275
	Plate 3	42	42	38	40	40	40	224
A.H.R± S.D		43.00±1.00	40.67±1.53	39.33±1.53	38.67± 1.53	39.67±0.58	37.67± 2.08	247.33 ± 25.77
TA 98	Plate 1	51	42	39	41	39	37	440
	Plate 2	52	41	41	38	40	39	465
	Plate 3	51	44	42	40	41	38	334
A.H.R ± S.D		51.33±0.58	42.33± 1.53	40.67±1.53	39.67± 1.53	40.00±1.00	38.00±1.00	413.00±69.55
TA 1537	Plate 1	18	15	16	15	14	14	238
	Plate 2	17	16	15	15	14	11	241
	Plate 3	17	15	13	14	15	13	225
A.H.R ± S.D		17.33±0.58	15.33± 0.58	14.67±1.53	14.67±0.58	14.33±0.58	12.67±1.53	234.67±8.50

Table: 1 with metabolic activation (S9)

Group		Solvent Control	Concentrations µg/plate					Positive Control
			312.25	625	1250	2500	5000	
TA 100	Plate 1	218	213	205	206	208	205	920
	Plate 2	218	213	208	207	209	204	854
	Plate 3	212	208	213	208	212	209	849
A.H.R± S.D		216.00±3.46	211.33±2.89	208.67±4.04	207.00±1.00	209.67±2.08	206.00±2.65	874.33±39.6
TA 102	Plate 1	346	337	340	340	334	328	943
	Plate 2	347	339	338	337	328	340	974
	Plate 3	351	341	332	318	339	322	840
A.H.R ± S.D		348.67 ± 2.08	348.00±2.65	339.00±2.00	336.67± 4.1	331.67±11.93	333.67±5.51	330.00±9.17
TA 1535	Plate 1	44	42	39	41	40	38	323
	Plate 2	46	41	42	41	38	39	404
	Plate 3	42	44	41	36	40	40	341
A.H.R± S.D		43.00±1.00	44.00±2.00	42.33±1.53	40.67±1.53	39.33±2.89	39.33±1.15	39.00±1.00
TA 98	Plate 1	52	40	43	40	42	39	241
	Plate 2	50	39	39	40	41	40	274
	Plate 3	49	42	40	38	40	42	224
A.H.R ± S.D		51.33±0.58	50.33±1.53	40.33±1.53	40.67± 2.08	39.33±1.15	41.00±1.00	40.33±1.53
TA 1537	Plate 1	18	15	16	15	14	14	238
	Plate 2	17	16	15	15	14	11	241
	Plate 3	17	15	13	14	15	13	225
A.H.R ± S.D		17.33±0.58	15.33± 0.58	14.67±1.53	14.67±0.58	14.33±0.58	12.67±1.53	234.67±8.50

Table: 2 without metabolic activation

#Concentration (µg/ml)		Total no. of cells scored	CBPI#	% Cytostasis	Total no. of BN scored	%MNi-BN*
Solvent control (Sterile Millipore water 50 µl/5 mL)	4h +S9	1000	1.98	-	2000	0.85
	4h -S9	1000	1.98	-	2000	1.00
	Continuous -S9	1000	2.01	-	2000	0.80
1250	Continuous -S9	1000	1.99	1.98	2000	1.05
2500	Continuous -S9	1000	1.96	4.95	2000	1.15
5000	Continuous -S9	1000	1.92	6.13	2000	1.00
1250	4h + S9	1000	1.91	7.14	2000	1.10
2500	4h + S9	1000	1.96	4.95	2000	1.15
5000	4h + S9	1000	1.93	5.10	2000	1.20
1250	4h - S9	1000	1.92	6.12	2000	1.30
2500	4h - S9	1000	1.79	19.38	2000	1.30
5000	4h - S9	1000	1.80	18.37	2000	1.30
CYP (5 µg/ml)	4h + S9	1000	1.46	53.06	2000	2.70
MMC (0.8 µg/ml)	4h - S9	1000	1.53	45.92	2000	3.40
	Continuous -S9	1000	1.56	44.55	2000	3.70
Colchicine (0.4 µg/ml)	4h -S9	1000	1.52	46.94	2000	3.95
	Continuous -S9	1000	1.53	47.52	2000	4.30

Table : 3Summary on *in vitro* micronucleus assay in human lymphocytes(72 h harvest)

+S9 - with S9; -S9 - without S9; BN - Binucleate cells; MNi-BN - micronucleated binucleate cells

- Culture duplicates set

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