A Study of Genotoxicity of Food Colourant "Sudan I" in White Mice

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Abstract:

The genotoxic effect of food colorant Sudan I was investigated using different cytogenetic parameters. Three doses of sudan I 0.325, 0.65 and 1.3 mg/kg.b.wt. which represent (Accepted Daily Intake ADI, double of the ADI, and four times of the ADI) were investigated. The doses were dissolved in distilled water and orally administrated and tested for the induction of SCE's, CAs, in bone marrow cells and primary- spermatocytes. Cyclophosphamid were used as positive control. The results showed statistically significant increase in SCEs, CAs in both bone marrow cells and primary- spermatocytes
In conclusion the investigators suggest that the use of this substance must be banned like what happened in many countries.

دراسة السمية الوراثية لملون الطعام " Sudan I "
في الفئران البيض

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المستخلص:

تمت دراسة السمية الوراثية لملون الطعام سودان باستخدام مقاييس مختلفة للوراثة الخلوية. تم اختبار ثلاث جرعات من الملون سودان (٠,٣٢٥، ٠,٦٥و ١،٣ ملغم\كغم. وزن جسم وهي تمثل (الجرعة المسموح بها يوميا وضعف الجرعة المسموح بها يوميا واربعة اضعاف الجرعة المسموح بها يوميا). تم تذويب الجرع بالماء المقطر واعطيت عن طريق الفم وتم اختبارها بالنسبة لحث تبادلات الكروماتيدات الشقيقة والشذوذ الكروموسومي في خلايا نقي العظم والخلايا الابتدائية المولدة للنطف. تم استخدام العقار سيكلوفوسفاميد كسيطرة موجبة. اظهرت النتائج زيادة معنوية احصائيا في تبادلات الكروماتيدات الشقيقة والشذوذ الكروموسومي في كل من خلايا نقي العظم والخلايا الابتدائية المولدة للنطف. ويستنتج الباحثان ان استعمال ملون الطعام سودان يجب ان يمنع كما جرى في العديد من الدول.

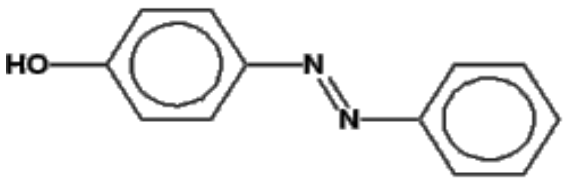
Introduction

Food additives which are essentially synthetic, have been a recent source of concern to the public as potential causes of various human diseases. They may be among factors responsible for the outbreak of cancer, hepatic and nephritic failures. Food additives are distinguished into two broad categories, direct and indirect (1) . Much attention has recently been brought to the fact that some food additives may have mutagenic potential (2,3).

Direct food additives, are intentionally added to our food supplies in small amounts and function to improve the appearance, texture and/or the quality of the product or to aid in its processing. The additives are also used to preserve and to improve the storage capability of food. This include *colouring agents, preservatives, emulsifiers, stabilizers, synthetic flavors and antioxidants* (4).
Some of the primary reasons of using food colors include:

- **Offsetting color loss due to light, air, extremes of temperature, moisture, and storage conditions.**
- **Masking natural variations in color.**
- **Enhancing naturally occurring colors.**
- **Providing identity to foods.**
- **Protecting flavors and vitamins from damage by light.**
- **Decorating purposes such as cake icing (5)**

Azo compounds refer to synthetic inorganic chemical compounds bearing functional group R-N = N – R , in which Rand R can be either aryl or alkyl. The N = N group is called an azo or diimide (6)



A typical Azo compound, 4-hydroxyphenylazobenzene, a.k.a. yellow azo dye

After oral intake, azo dyes can be reduced to the corresponding amines. Azo reduction can take place by means of reductase of the

gastrointestinal microflora and also through microsomal and cytosolic reductase of the liver and extra-hepatic tissue. Gastrointestinal microflora plays a major role here (7).

The mutagenicity determined in numerous cases in *in vitro* test systems and the

carcinogenic action in animal experiments are attributed to the release of amines and their ensuing metabolic activation. This leads to the suspicion that all azo compounds containing a carcinogenic amine component which can be released during metabolism, have carcinogenic potential (8).

Sudan I is classified as a category 3 carcinogen and as a category 3 mutagen in Annex I of the Directive 67/548/EC. It is not listed in the Technical Rules for Dangerous Substances (9). The classification recommendation of the Committee for Dangerous Substances (AGS) corresponds to the classification in Annex I of the Directive 67/548/EC.

AGS gave the following reasons for its classification recommendation:

C.I. Solvent Yellow 14 (Sudan I) led to a dose-related higher incidence of neoplastic liver nodules in rats after administration in feeding. The cases of leukaemia and lymphoma, which occurred in mice, do not show any clear dose dependency and are, in some cases, in the range of control values (10).

After oral administration, the substance led to a higher rate of micronuclei in polychromatic erythrocytes in rats whereas in mice the micronucleus test was negative or slightly positive.

Because of the neoplastic liver nodules that occurred in rats, which are deemed to be cancer precursors, and the genotoxic efficacy *in vivo*, C.I. Solvent Yellow 14 should be classified as a category 3 carcinogen and a category 3 mutagen. When it comes to reproduction toxic effects, the substance cannot be classified since no data are available (11),

Because of the skin-sensitizing effect in test animals and in humans, the substance should be classified as skin sensitizing (R43) (12).

Material and Methods

Animals

White Swiss mice were used in all experiments. The animals were obtained from closed random bred colony at the Iraqi Research Center of Cancer and Medical Genetics, maintained under controlled conditions of temperature and humidity and received food and water ad libitum.

Test Chemical

Sudan I 842-07-9 12055 1-phenylazo-2-naphthol 1-phenylazo-ß-naphthol 2-hydroxy-1-phenylazonaphthalene 2-hydroxynaphthyl-1-azobenzene Solvent Yellow 14 Sudan Gelb Dispersol Yellow PP Ölorange E Scharlach B

Treatment and Cytological Preparations

The doses were dissolved in distilled water, and orally administered. The method described by Allen (13), for *in vivo* SCE's induction in mice bone-marrow was applied with some modifications (14).The method of Yosida and Amano (15) was used for chromosome aberration in bone marrow cells with some modifications. For Chromosomal abnormalities in spermatocytes the method of Evans *et al.* (16) was used. Mice were orally treated with successive daily dose of sundan I at the dose of 0.325mg/ kg b.wt. for 3 weeks. In all experiments, the significance of the experimental from control data was calculated using t-test according to Ronald *et al.* (17). Significant ($P < 0.05$), Highly significant ($P < 0.01$).

Sister Chromatid Exchange

Mature male mice aged 9-12 weeks and weighing 25-30g were used. Three different dose levels of Sudan I (0.325, 0.65 and 1.3 mg/kg.b.wt.) were tested. Based on protocol of (13) and I.P. injection is appropriate for studying SCEs in mouse bone marrow. 5-Bromodeoxyuridine BrdU, (Sigma). Tablets weighing approximately 55±5mg were placed subcoutaneously for 21 hours. Mice were injected I.P. Sudan I dissolved in distilled water 8 hours following BrdU treatment, and with colchicine 0.6 mg/kg[1]b.wt. 2 hours prior to killing. Metaphase spreads were prepared and stained according to the fluorescence 33258 Hoechst dye plus Giemsa method.

Five mice were used for each treatment and 25 well spread metaphases per animal were examined microscopically for scoring SCEs in order to evaluate the differences in mean SCE frequencies between treated and control groups using t-test.

Chromosomal Aberrations

For bone marrow or spermatocytes, the experimental design is based on studying the effect of Sudan I by oral giving for 3 weeks at different dose levels (0.325, 0.65 and 1.3 mg/kg.b.wt.) The highest tested dose 1.3mg/kg.b.wt. was used for studying the effect of Sudan I at different time intervals (1,2, and 3 weeks). Chromosomes from bone marrow cells were prepared following the method of Yoshida and amino (15). Spermatocytes metaphases at diakinesis metaphases I were prepared according to Evans *et al* (16). Slides were stained with 7% Giemsa in phosphate buffer pH6.8. At least 75 well spread metaphases were analyzed per each animal for scoring different types of aberrations. Statistical evaluation was performed using t-test.

Statistical Evaluation

The significance of the experimental from control data was calculated using student t-test. Significant values P< 0.05 and highly significant P< 0.01.

Results

Sister- Chromatid Exchanges:-

Frequency of SCEs expressed per cell. The control value was obtained from 125 metaphase of bone-marrow cells treated with BrdU. Figure (1) represents a metaphase with SCEs from non-treated bone-marrow cells (negative control).

Fig. 1 Metaphase spreads of whit mice in control group shows sister chromatid exchange. Heochst + Giemsa stain 100x.

Table (1, 2) represents effects of cyclophosphamide and Sudan I on the induction of SCEs in bone-marrow cells. Frequency of SCEs reached 34.55± 1,46/ cell which is highly significant (p< 0.01)

compared with 2.4± 1.o5 for the control of non-treated animals. Metaphase with different types of SCEs (single, double,triple and quadruple) were observed (Table-2).

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Table 1. Frequency of sister Chromatid Exchanges (SCEs) in Mice Bone-marrow Cells.

Treatment and doses Mg/kg.b.wt.	No. of mice	No. of examined metaphases	No. of SCEs	SCEs/ cell Means± S.E.
I. Control (non treated)	5	125	525	4.2± 1.05
II. Cyclophosphamide (positive control)				
20	5	125	4319	34.55± 1.46**
III. Sudan I				
0.325	5	125	680	5.44± 1.62*
0.65	5	125	956	7.65± 0.58*
1.3	5	125	1055	8.44± 0.7*

** significant at p< 0.01 (t-test)

Table 2. Number and Percentage of Chromosomes with Single, Double, Triple and Quadruple Sister Chromatid Exchanges (SCEs) in Mice Bone-marrow Cells.

T/ D Mg/kg.b.wt.	No. of chromosomes	Single SCEs		Double SCEs		Triple SCEs		Qudruple SCEs	
		No.	%	No.	%	No.	%	No	%
I. Control (N.T)	5000	385	7.7	44	0.88	-	-	-	-
II.Ctclophosphmide 20	5000	1020	20.4	510	10.2	187	3.74	97	1.94
III. Sudan I									
0.325	5000	487	9.74	58	1.16	1	0.02	-	-
0.65	5000	678	13.56	79	1.58	8	0.12	-	-
1.3	5000	756	15,12	86	1.72	5	0.1	-	-

Chromosome Aberrations in Bone-Marrow Cells:-

Fig. 2 shows metaphase spread of white mice in control group which has 40 metaphase chromosomes.

Fig. 2 Metaphase spread of whit mice in control group shows 40 chromosome. Giemsa stain 100x

The results of the present study demonstrated that sudan I is a potent inducer of chromosomal aberrations in bone- marrow cells and spermatocytes. The percentage of metaphases with chromosomal aberration in bone- marrow was found to be statistically significant at 24 and 48 hrs after oral administration of three doses of sudan I 0.325, 0.65 and 1.3 mg/kg.b.wt. for five consecutuive days tree succesive weeks.

Tables (3, 4) show chromosomal aberrations induced in bone-marrow cells 24hrs after oral administration of three different doses of Sudan I for five consecutive days three succesive weeks. All the tested concentrations of Sudan I induced statistically significant percentage of chromosomal aberrations. Such percentage was found to be dose-dependent.

Table 3. Number and Percentage of metaphases with Chromosomal Aberrations Induced in Mice Bone-marrow Cells 24hrs after Oral Administration of Three Different Doses of Sudan I for Five Consecutive Days three Succesive weeks.

T/ D Mg/kg.b.wt	No. of Mice	No. of Examined Metaphases	No. of Abnormal Metaphases	Abnormal Metaphases	
				Including Gaps	Excluding Gaps

				Mean± S.E.	Mean± S.E.
I. Control (N.T.)	5	375	9	2.4± 0.2	1.3± 0.2
II. Cyclophosphamid 20	5	375	132	35.2± 0.7**	28.5± 0.31**
III. Sudan I 0.325	5	375	55	14.66± 0.81**	9.33± 0.89**
0.65	5	375	73	0.63**	12.8± 0.81**
1.3	5	375	91	19.46± 24.26± 0.2**	12.8± 0.24**

** significant at p< 0.01 (t-test)

Table 4. Number and Percentage of the Different Types of Chromosomal Aberrations Induced in Mice Bone-marrow Cells 24hrs after Oral Administration of Different Doses of Sudan I for Five Consecutive Days three succesivs weeks.

T/ D Mg/ kg.b.wt	No. of mice	No. of Examined metaphases	No. and percentage of metaphases with:											
			Chromatid and/ or Chromos. Gap		Fragments and/ or Breaks		Deletion		R.T.		Aneuploid		Euploid	
			N0.	%	No.	%	No.	%	No.	%	No.	%	No.	%
I. Control (N.T.)	5	375	4	1.06	3	0.8	2	0.53	-	-	-	-	-	-
II. Sudan I 0.325	5	375	20	5.33	16	4.26	13	3.46	-	-	4	1.06	-	-
0.65	5	375	25	6.66	34	9.06	8	2.13	1	0.26	1	0.26	2	0.53
1.3	5	375	43	11.46	36	9.6	4	1.06	-	-	-	-	3	0.8

Chromosome Aberrations in Primary-Spermatocytes:-

Fig. 3 shows metaphase chromosomes in diakinesas which represent 19 autosomal bivalents and 1 X -Y bivalent.

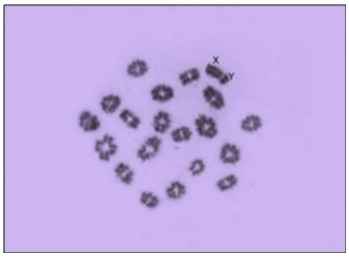

Fig. 3 Diakinesis chromossomes of Whit Mice in Control Group shows 19 Autosomal and 1 X-Y Bivalent. Giemsa stain 100x

The effects of oral administration of Sudan I for five consecutive day three succesive weeks in induction of chromosomal abnormalities in

mouse primary-spermatocytes at diakinesis-metaphase I are presented in tables (5, 6).
All the tested doses of Sudan I (0.325, 0.65 and 1.3 mg/ b.wt.) induced a statistically significant dose dependent increase in percentage of chromosomal abnormalities in mice primary-spermatocytes.

Table 5. Number and Percentage of Metaphases with Chromosomal Abnormalities Induced in Mice Primary-Spermatocytes 24hrs after Oral Administration of Different Doses of Sudan I for Five Consecutive Three Successive Weeks.

T/ D Mg/kg.b.wt	No. of mice	No. of Examined Metaphases	No. of Abnormal Metaphases	
I. Control (N.T.)	5	500	12	2.4± 0.24
II. Cyclophosphamid 20	5	500	50	10.0± 0.54**
III. Sudan I				
0.325	5	500	32	6.4± 0.4**
0.65	5	500	44	8.8± 0.49**
1.3	5	500	79	15.8± 0.73**

** Significant at p< 0.01 (t-test)

Table 6. Number and Percentage of the different Types of Chromosomal Abnormalities Induced in Mice Primary-Spermatocytes 24hrs after Oral Administration of Different Doses of Sudan I for Five Consecutive Three Successive Weeks.

T/ D Mg/kg.b.wt.	No. of mice	No. of Examined metaphases	No. and percentage of metaphases with:							
			X-Y univalent		Autosomal univalent		Chain IV		Polyploid	
			No.	%	No.	%	No.	%	N0.	%
I. Control (N.T.)	5	500	8	1.6	4	0.8	-	-	-	-
II. Sudan I										
0.325	5	500	14	2.8	15	3	-	-	-	-
0.65	5	500	18	3.6	22	4.4	4	0.8	-	-
1.3	5	500	49	9.8	30	6	-	-		-

Discussion

The wide use of food additives has evolved a great controversy about its effect on human body. The cytogenetic effect is one of the

important factors which needs careful and deep investigation since it does not only affect the user, but also the generations.

Since the inception of the discipline of mutagenesis in eukaryotes, it has been recognized that structural changes induced in chromosomes constitute a significant proportion of the mutagenic events. The past two decades had a chieved prodigious progress in the area of mammalian germ cell cytogenetics.

In general, to produce chromosomal aberrations, the chemical mutagen must be present at the time when the cell is replicating its DNA, so that the lesion introduced into the DNA can be converted into chromosome aberration which may or may not be transmitted to the daughter cells.

In the present work, a study has been conducted to determine the potential genotoxic effects of the food additive "sudant I in somatic and germ cells of mice in vivo using different mutagenic end points.

The study included: the effect of sudan I on the induction of sister chromatid exchanges in bone-marrow cells, chromosomal aberration analysis in somatic (bone marrow) and germ cells (primary-spermatocytes) after repeated oral treatments.

The in vivo tests should be the primary tests for detecting cytogenetic abnormalities induced by drugs, food additives, metals, pesticides or other chemical agents to which the public is exposed. The prime reasons for this are that this approach combines the relevance of a mammalian system and the possibility of detecting abnormalities produced by metabolic products of the agent administered, as well as by the original agent (18).

A concurrent positive control group should be included for each experiment. In the present study, cyclophosphamide was used as a positive control. The drug was i. p. injected and samples were taken 24hrs post treatment. It is acceptable that a positive control may be administered by a route different from the test agent and sampled at only a single time (19).

The antineoplastic drug cyclophosphamide (CP) is a commonly used chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic diseases and some autoimmune diseases. (20).

Many studies have demonstrated that the alkylating agent cyclophosphamide causes gene mutation, chromosomal breakage,

rearrangements, and aneuploidy in somatic cells. It also increased the frequency of secondary treatment-related tumors in human cancer survivors (21). Moreover, animal studies have shown clear evidence that cyclophosphamide causes injury to germ cells as well as induction of transmissible genetic damage (22).

Sister chromatid exchange test was used to assess the mutagenic potential of sudan I. Sister chromatid exchange (SCE) has proved to be a highly sensitive indicator for assessing suspected mutagens or carcinogens. SCE's can be precisely scored and readily induced in mammalian cells by various known mutagenes and carcinogens at concentrations far below cytotoxic and clastogenic doses (23).

The generation of sister chromatid exchange is dependent on homologous recombination and is elevated in a number of circumstances including following exposure to DNA damaging agents and in some genetic backgrounds that result in increased dependence on recombination based pathways (24).

In the present study, sudan I induced a significant increase in the frequency of SCE's in mouse bone-marrow cells with a dose-related relationship. The maximum frequency of SCE's/ cell reached 8.44±0.7 and 9.23±0.31 after treatment with the highest dose of sudan I Such values were significantly lower than that induced by the positive control "cyclophosphamide" (34.55±1.46).

Due to the exposure to various potentially genotoxic chemicals, the chromosome aberrations were considered as bioindicators of environmental genotoxicity (25,26).

In the present work, repeated oral treatment with different doses of sudan I induced a significant percentage of aberrations in mouse bone-marrow cells as well as in mouse spermatocytes with a dose-related relationship. Such results coincide well with the results of other authors who proved the clastogenicity of some food additives in mammalian cells (27).

It is worth mentioning that the percentage of the induced chromosomal aberrations after oral treatment of animals with different doses of sudan I is higher in somatic cells (bone marrow) than that recorded in germ cells (primary-spermatocytes). A similar phenomenon was also observed by other mutagens (28).

This may be explained by the fact that somatic cell test is more sensitive than germ cell test (29,30). Moreover, the levels of chemicals in the blood stream in the systemic circulation can be used

to approximate somatic cell exposure, but are not necessarily accurate for germ cell exposure since the gonads are protected from the general circulation by what are referred to as blood barriers which probably reduce the risk of exposure of germ cells compared to somatic cells (31).

The correlations between male sterility and alterations in the organization of the sex chromosome cores and X-Y chromatin may indicate that impaired signals from the XY domain may interfere with the progression of the primary- spermatocyte through the prophase (32).

The present results indicate that sudan I is genotoxic in the tests examined. Sudan Iinduced a significant frequency of SCE's and chromosomal aberrations in vivo in mouse bone marrow and primary-spermatocytes after repeated oral treatments.

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