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# Assessment of Potential Mutagenic Effect of Colorant of Some Commercial Fruit Drinks in Mice

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## Abstract

Due to the large consumption of commercial fruit drinks worldwide in recent years and considering that some of the components present in their composition cause potential risks to human health, therefore, this study was designed to evaluate the potential mutagenic effect of colorants of commercial fruit drinks (pear, cherry, strawberries and red grape) stored at 4°C for six months, on mice using comet assay, DNA fragmentation , and micronucleus test as a good indicators for strand breaks in DNA, as well measuring the malondialdehyde (MDA) level. Three doses 0.8, 1.6 and 2.4 mg/kg bw of 4 commercial fruit drinks were administered orally for mice for 3 weeks beside the control group. Mice were sacrificed 24 hours after the last dose and subjected to micronucleus and comet assays as well DNA degradation and MDA analysis. The results revealed that a significant increase in tail length of comet percentages from blood cells as well in the frequency of micronucleated cells (MNCs) and DNA fragmentation following administration of commercial fruit drinks was achieved compared to control group. The level of MDA was increased ($P<0.05$) significantly after administration of commercial fruit drinks especially with the high dose (2.4 mg/kg bw) of treatment compared to control. In conclusion, this study serves as a warning about the consumption of commercial fruit drinks and for the need for further studies in order to evaluate the long term mutagenic effect of these colorants on human health since some soft drinks are consumed daily by a significant proportion of the world population.

**Keywords:** Mutagenic; Effect; Colorant; Anthocyanin; Storage; Fruit drinks; Mice

## Introduction

Due to the development of modern techniques, which aim to increase the production, preservation, and packaging and improve certain properties, such as color and taste of foods, some substances in certain foods can induce mutations in the genetic material and may favor the development of tumors [1].

Colorants play important roles in food industry by providing enhancement, imitation or masking of the natural color of food products. Synthetic colorants have been widely used in food and related industry. However, concerns regarding their harmfulness to human's health are rising because of their potential toxicity [2,3]. This has led to the search for alternative natural colorants that are derived from plants and microorganisms [4]. The main groups of natural food colorants are carotenoids, chlorophylls, betacyanins, curcuminoids, and anthocyanins [5]. They are generally regarded as safe and preferable for their potential nutritional and therapeutic benefits [6].

Anthocyanins have been recognized since the early decades of this century as responsible for many of the beautiful red and blue colours of flowers and fruits [7-9]. They can be isolated from the plants in the red coloured flavylium form, which is present only at very acidic pH values (pH</1).

Anthocyanins are generally used as natural colorants especially for reddish to purplish hues. They are collectively the largest group of water-soluble pigments in the plant kingdom and have been used as food additives in various traditional cooking and food industry [10]. Anthocyanins have attracted considerable interest because of their biological properties as antioxidants, antitumor, anti-inflammatory and cardio-protective agents [11]. Regarding the safety of anthocyanins, the Joint FAO/WHO Expert Committee on Food Additive (JECFA) approved that anthocyanins are of a very low order of toxicity based on a number of tests including mutagenicity, reproductive toxicity, teratogenicity and acute and short-term toxicity evaluations [12]. However anthocyanins dissappear as monomeric compounds and are transformed into polymeric forms. This transformation results in a color change to a more brownish shade [13]. Storage temperature and storage time is the main responsible factor for anthocyanin loss [14,15]. Degradation rate increases as corresponding with food properties and anthocyanins compositions. The importance of the assessment of the effect of colorants (anthocyanine drinks) upon the genetic make up of mankind is obviously a critical issue in the use of these chemicals. An increasing of commercial drinks, which contain food colorants for a long periods are shown to exhibit genotoxic effects. Therefore, they possessed potential hazards to the human health [16].

Also, anthocyanin pigments may play a role in the prevention of oxidative damage in living systems. However, anthocyanin and PCA have been shown to have antioxidant activity and to offer protection against atherosclerosis and cancer [14], DNA is a vital molecule in the cell activities and was the main target for chemicals induced cell injury. The commercial pigment or coloring agent has been implicated in several clinical conditions, but most experimental work has concentrated on childhood hyperactivity, articaria, asthma [15]. Wojewodzka et al. [17] consider inter individual variability important; it can be detected by the analysis of parameters in the comet assay. They found considerable intra-individual homogeneity, and high inter individual variability, suggesting that the extent of the damage, as well as the decrease in the

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capacity of DNA damage repair, constantly induced by endogenous or exogenous factors, may be involved in the variability of the individual responses found.

With respect to the color additive present in the Grape-flavored soft drink, several results obtained corroborate the mutagenicity negative results. Poul et al. [18] showed that the acute oral exposure to food dye additives amaranth and tartrazine, present in this soft drink, did not induce genotoxic effects in the intestine of mice as in the micronucleus test. Al- Mossawi [19] showed that amaranth should be considered a weak mutagen. Clode et al. [20] showed that at doses up to 1250 mg/kg bw/day in rats, this colorant showed no adverse effects on fertility, hematological parameters, and serum chemistry or tumor incidence. Borzelleca and Hallagan [21] also showed that tartrazine was not toxic or carcinogenic in a chronic study on rats. Furthermore, the dye Brilliant Blue FCF, another constituent of the Grape-flavored soft drinks, did not present mutagenic in the Ames test [22], and was not toxic or carcinogenic in rats and mice [23].

The study of Düsman et al. [24] serves as a warning about the consumption of Cola and Grape-flavored soft drink, which showed mutagenic potential in the bone marrow of Wistar rats treated *in vivo*. However, further studies are required to evaluate the cytotoxic and mutagenic long term effects of these colorants are recommended since some fruit drinks are consumed daily by a significant proportion of the world population.

Therefore, this study was designed to evaluate the potential mutagenic effect of colorants of commercial fruit drinks in mice using micronucleus and comet assays as well DNA degradation and MDA analysis.

## Materials and Methods

### Animals and experimental design

Swiss albino male mice weighting about (25-30 g) obtained from a closed random-bred colony at the National Research Center, Cairo, Egypt, were used. Food and water were provided *ad libitum* (means regularly sufficient food and water). Mice were divided into 5 groups, the 1st group served as control and the 2nd, 3rd, 4th and 5th groups were administered with pear, cherry, strawberry and red grape, respectively. Three doses 0.8, 1.6 and 2.4 mg/kg bw of commercial fruit drinks were administered orally for the treated mice for 3 weeks for consecutive days and mice were sacrificed 24 hours after the last dose and subjected to micronucleus and comet assays and DNA fragmentation as well MDA analysis.

### Commercial fruit drinks

Commercial fruit drinks (pear –*'Alexander Lucas'*, cherry – *(Prunuscerasus L.*), strawberry – (*Fragaria x ananassaDuch.*) and red grape –*Vitisvinifera L*) with colorant (# RC1539) were obtained from the International Frutta Labs Co., Industrial Zone – 6th October City, Egypt. The fruit drinks had been filled into 200 ml glass bottles, stored and evaluated as the other commercial drinks. Upon production, the samples arriving to the laboratory were stored at refrigerator temperature (approximately +4°C) for six months in darkness.

#### Micronucleus assay

Mice were sacrificed and both femurs of mice were removed and bone marrow was aspirated with fetal calf serum. The bone marrow smears were made, fixed and stained with Giemsa and 2000 polychromatic erythrocytes were examined per animal for all groups according to Valette [25].

#### Comet assay

The comet assay was carried out under alkaline conditions as described by Singh [26]. Images of 100 randomly selected cells from each dose (0.8, 1.6 and 2.4 mg/kg bw of the 4 commercial fruit drinks used in the study) were analyzed at 400X using fluorescence Microscope with attached camera. Comets were classified and the percentage of comet cells was calculated.

#### Biochemical analysis

Malondialdehyde determination (MDA)

Liver was homogenized and the supernatant was chemically treated and centrifuged at 10000 rpm for 3 min for quantitative measurement of lipid peroxidase malondialdehyde (MDA) according to the method of Ohkawa [27].

### Statistical Analysis

Data were analyzed statistically using one way analysis of variance (ANOVA), least significant difference (LSD) and correlation coefficient (square root) as described by Richard [28]. Statistical analysis was performed using MS Excel XP software.

## Results and Discussion

### Mutagenic effect of some commercial fruit drinks

The results of the mutagenic **effect of some commercial fruit drinks**on Swiss albino male mice, ingested orally with pear, cherry, strawberries and red grape (0.8, 1.6 and 2.4 mg/kg bw) were listed in Tables 1-3 using micronucleus and comet assays and DNA fragmentation.

| Fruit Drinks | Micronucleus frequency (Mean+SD) | | |
|---|---|---|---|
| | Dose | | |
| | (0.8 mg/kg bw) | (1.6 mg/kg bw) | (2.4 mg/kg bw) |
| Control | 6.8 ± 0.80[a] | 6.80 ± 0.80[a] | 6.8 ± 0.80[a] |
| Pear | 43.70 ± 0.53[e] | 48.20 ± 2.35[e] | 52.7 ± 0.45[e] |
| Cheery | 23.0 ± 0.33[c] | 28.40 ± 0.74[c] | 34.10 ± 0.4o[c] |
| Strawberry | 28.4 ± 0.31[d] | 34.05 ± 0.70[d] | 39.70 ± 0.66[d] |
| Red grape | 8.50 ± 0.31[b] | 12.80 ± 0.37[b] | 16.90 ± 0.45[b] |

Small different superscript letters are differing significantly.

**Table 1:** Effect of colorant of some commercial fruit drinks on micronucleus frequency in bone marrow cells of mice (n=5).

| Fruit drinks | Dose (mg/kg bw) | No of cells | | Comet class | | | | DNA damaged cells (%) |
|---|---|---|---|---|---|---|---|---|
| | | Analyzed | Comet | 0 | 1 | 2 | 3 | |
| Control | | 100 | 5 | 95 | 4 | 1 | 0 | 5 |
| Pears | 0.8 | 100 | 52 | 48 | 19 | 17 | 16 | 52 |
| | 1.6 | 100 | 57 | 43 | 20 | 19 | 18 | 57 |
| | 2.4 | 100 | 63 | 34 | 22 | 21 | 20 | 63 |
| Cheery | 0.8 | 100 | 22 | 78 | 8 | 9 | 5 | 22 |
| | 1.6 | 100 | 27 | 73 | 10 | 10 | 7 | 27 |
| | 2.4 | 100 | 33 | 67 | 12 | 11 | 10 | 33 |
| Strawberry | 0.8 | 100 | 38 | 62 | 15 | 12 | 11 | 38 |
| | 1.6 | 100 | 43 | 57 | 16 | 14 | 13 | 43 |
| | 2.4 | 100 | 49 | 51 | 18 | 16 | 15 | 49 |
| Red grape | 0.8 | 100 | 9 | 91 | 4 | 3 | 2 | 9 |
| | 1.6 | 100 | 12 | 88 | 5 | 4 | 3 | 12 |
| | 2.4 | 100 | 15 | 83 | 6 | 5 | 4 | 15 |

**Table 2:** DNA damage frequency in mice treated with some commercial fruit drinks using comet assay.

| Fruit drinks | DNA Fragmentation (Mean ± SE) | | |
|---|---|---|---|
| | Dose | | |
| | (0.8 mg/kg bw) | (1.6 mg/kg/bw) | (2.4 mg/kg bw) |
| Control | 5.8 ± 0.37[a] | 5.8 ± 0.37[a] | 5.80 ± 0.37[a] |
| Pear | 52.40 ± 0.60[e] | 57.80 ± 1.15[e] | 63.40 ± 0.60[e] |
| Cherry | 22.80 ± 0.96[c] | 28.20 ± 0.86[c] | 33.80 ± 0.37[c] |
| Strawberry | 38.00 ± 0.54[d] | 43.80 ± 3.20[d] | 49.60 ± 0.81[d] |
| Red grape | 9.60 ± 0.24[b] | 12.40 ± 0.67[b] | 15.40 ± 0.24[b] |

Small different superscript letters are differing significantly.

**Table 3:** Effect of colorant of some commercial fruit drinks on DNA fragmentation in mice.

| Fruit drinks | Malondialdhye (MDA) level (Mean ± SE) | | |
|---|---|---|---|
| | Dose (mg/kg Bw) | | |
| | (0.8 mg/kg bw) | (1.6mg/kg bw) | (2.4 mg/kg bw) |
| Control | 0.66 ± 0.210[a] | 0.66 ± 0.210[a] | 0.66 ± 210[a] |
| Pear | 21.16 ± 0.477[e] | 25.40 ± 0.50[e] | 29.83 ± 0.401[e] |
| Cheery | 12.0 ± 0.365[c] | 14.40 ± 0.50[c] | 17.0 ± 0.365[c] |
| Strawberry | 14.50 ± 0.223[d] | 17.40 ± 0.50[d] | 20.50 ± 0.562[d] |
| Red grape | 2.33 ± 0.333[b] | 5.80 ± 0.37[b] | 9.50 ± 0.557[b] |

Small different superscript letters are differing significantly.

**Table 4**: Effect of colorant of some commercial fruit drinks on lipid peroxidase malondialdhyde (MDA) level in mice (n=5).

### Micronucleus assay

To evaluate the mutagenicity *in vivo*, we employed micronucleus assay in mice ingested with commercial fruit drinks (Table 1). Throughout the assay, there was a statistically significant difference ($P>0.05$) between the number of micronucleated peripheral reticulocytes (MNRETs) of treated and control mice ($P<0.05$). Our result indicated that the treatments with stored commercial fruit drinks induced an elevation of micronucleus generation in mice. This is consistent with the previous study demonstrating positive mutagenicity of colorants, in *in vivo* comet and micronucleated reticulocyte assays in mice [29]. As well this is in accordance with the tests of commercial proanthocyanidin-based colorants that were tested positive inin vitro mutagenicity tests but do not confer in vivo mutagenicity [30].

However, a study on in vivo micronucleated reticulocyte assay revealed that no mutagenicity was observed up to 1 g/kg bw of anthocyanin-based pigment extract [31].

DNA is a target for mutagens and carcinogens, which induce changes in DNA structure giving rise to mutations and/ or cell death [32]. In the present study DNA assay damage was evaluated by comet (Table 2) and micronucleus test. Administration of commercial fruit drinks resulted in DNA damage correspond to DNA from animals exposed to 0.8, 1.6 and 2.4 mg/kg bw fruit drinks, respectively. It is evident that exposure to fruit drinks resulted in DNA damage as compared to control. It is clear that extent of DNA damage is dose dependent. The mechanism of action is depend on the generation of reactive oxygen species (ROS), which could provide explanations of the different findings of the *in vivo* comet assay and micronucleus test observed in mice and in rats as reported by EFSA [33].

The results of genotoxicity of commercial fruit drinks assessed by scoring MN (Table 1), corroborate with the findings of comet assay (Table 2). A significant ($P<0.05$) increase in the frequency of micronuclei was observed after treatment with commercial drinks as compared to that in control. This is clear in all doses (0.8, 1.6 and 2.4 mg/kg bw) used in the study.

Sasaki et al. [34] studied the genotoxicity of 39 chemicals currently in use as food additives. They treated groups of four male ddY mice once orally with each additive at up to half its LD50 or the limit dose (2000 mg/kg bw) and performed the comet assays on the glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow 3 and 24 hours after treatment. Tartrazine induced dose- related DNA damage in the glandular stomach, colon, and/or urinary bladder. All 7 food dyes tested induced DNA damage in the gastrointestinal organs at low doses (10 or 100 mg/kg bw). Among them, Amaranth, Allura Red, New Coccine, and Tartrazine induced DNA damage.

The effect of commercial fruit drinks on DNA fragmentation in mice (Table 3) was clearly evident where there was a significant increase in DNA fragmentation in all treated groups compared to control in a dose dependent manner and that in agreement with Haveland [35] who studied the genetic effects of 25 colorants dyes belonging to 6 major structural classes and proved their abilities to cause DNA damage and mutations in bacterial assay systems. As well with those found by Borzelleca and Hagan [36] who showed that erythrosine, brilliant black and indigo carmine caused tumors in the different organs of rat and mice compared to control.

All commercial fruit drinks caused a highly significant increase in chromosome aberration in both bone marrow and spermatocyte cells. Sub-acute treatment caused high percentage of aberrant cells due to the accumulation effect of the commercial fruit drinks. Deletion is the main type of chromosomal aberrations in both types of examined cells (i.e. loss of the DNA content). Since DNA is considered as constant genetic component of every cell in all organs, the decrease of DNA content may be due to the presence of colorants in commercial fruit drinks, which caused cell hyperplasia or cell enlargement [16]. This suggestion is in agreement with our findings as well.

The levels of MDA in control and experimental mice are shown in Table 4. The level of MDA level was increased ($P < 0.05$) significantly after administration of commercial fruit drinks especially with the high dose (2.4 mg/kg bw) of treatment compared to control. Free radicals or reactive oxygen species (ROS) are responsible for oxidative stress [37]. Free radicals formed have a great potential to react rapidly with lipids, which in turn leads to lipid peroxidation (LPO). The level of malondialdehyde (MDA) has been widely used as a biomarker of lipid peroxidation (LPO) for many years [38].

In conclusion, the results obtained from *in vitro* comet assay, *in vivo* micronucleated reticulocyte assay, and DNA fragmentation presented in this study demonstrated that commercial fruit drinks consumption displays mutagenic effect compared to control. This study serves as a warning about the consumption of commercial fruit drinks and for the need for further studies in order to evaluate the long term mutagenic effect of these colorants on human health since some soft drinks are consumed daily by a significant proportion of the world population.

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