Genotoxic and mutagenic effects of erythrosine B, a xanthene food dye, on HepG2 cells

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A R T I C L E   I N F O

Article history:
Received 27 April 2012
Accepted 19 July 2012
Available online 28 July 2012

Keywords:
Comet assay
Food colorant
In vitro
Micronucleus test

A B S T R A C T

Erythrosine (ErB) is a xanthene and an US Food and Drug Administration approved dye used in foods, drugs and cosmetics. Although its utilization is permitted, ErB is described as inhibitor of enzymes and protein–protein interactions and is toxic to pituitary and spermatogenesis processes. However, the genotoxicity and mutagenicity of ErB is inconclusive in the literature. This study aimed to analyze the genotoxicity of this dye using the alkaline comet assay and is the first investigation to evaluate ErB mutagenicity using the cytokinesis block micronucleus cytome (CBMN-Cyt) assay in HepG2 cells. These cells were chosen because they produce phase I and phase II enzymes that can mimic in vivo metabolism. The cells were treated with seven concentrations (0.1–70.0 μg mL⁻¹) of ErB, and the results showed genotoxicity at the two highest concentrations and mutagenicity at six concentrations. Furthermore, as micronuclei result from clastogenic and aneugenic processes, while comet assay is often considered more sensitive and detects DNA single strand breaks, we suggest that an aneugenic is responsible for the observed damage. Although ErB is approved for use in the food, cosmetic and pharmaceutical industries, it must be used carefully because it damages the DNA structure.

1. Introduction

The use of color additives dates from 5000 B.C. by the Ancient Egyptians, and historians estimate that colored food emerged by 1500 B.C. Currently, the food industry considers color as an important criterion for food choice, thus synthetic colorants are frequently used to improve the esthetic quality (Mpountoukas et al., 2010). The literature data estimates the production of colorants to be 8,000,000 tons annually (Revankar and Lele, 2007).

The use of these artificial colorants would be safe if their consumption was below the ADI (Acceptable Daily Intake) limit. However, when consuming high amounts of these colored products, people may be exposed to high concentrations of these chemical compounds, especially children due to their low body weights compared to adults (Dixit et al., 2011).

Among the synthetic food dyes, erythrosine B (ErB, 2',4',5',7'-tetraiodofluorescein) is a cherry-pink food colorant with a polyiodinated xanthene structure; it is unique in this class approved by the US Food and Drug Administration (FDA) and is widely used in foods, drugs and cosmetics (Ganesan et al., 2011; Mpountoukas et al., 2010; Silbergeld and Anderson, 1982). Although its utilization is permitted, ErB has been described as having an influence on childhood behavior (Silbergeld and Anderson, 1982) and interfering with thyroid function due to the high iodine content (Bora et al., 1969; Jennings et al., 1990b). In vitro models showed the inhibition of drug-metabolizing enzymes (Mizutani, 2009), potent inhibition of two protein–protein interactions involving the tumor necrosis factor (TNF) superfamily (Ganesan et al., 2011) and both high cytotoxicity and cytostaticity (Mpountoukas et al., 2010).

However, there are conflicting results with regard to genotoxicity and mutagenicity. Both negative (Auletta et al., 1977; Brown et al., 1978) and positive (Matula and Downie, 1984) results were obtained in bacterial reversion assays, and Lakdawalla and Netrawali (1988b) and Lin and Brusick (1986) describe negative results using the Ames test. Therefore, the goal of the present study was to evaluate the genotoxicity and mutagenicity of ErB in vitro using the comet assay and cytokinesis-block micronucleus cytome (CBMN-Cyt) assay. We used HepG2 cells because this cell line pro-

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duces phase I and phase II enzymes, which are useful for reducing false-positive events (Kirsch-Volders et al., 2011).

2. Materials and methods

2.1. Chemical compounds and concentration selection

Erythrosine B (ErB; CAS, 15905-32-5; CI, 45430; ≥95% purity; Fig. 1), trypan blue (CAS: 72-57-1), cytochalasin B (CAS: 14930-96-2) and acridine orange (CAS: 10127-02-3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Doxorubicin (CAS: 22314-92-8) was purchased from Laboratório Bêrgamo (Taboão da Serra, Brazil). Dimethylsulfoxide (DMSO, CAS: 67-68-5) was purchased from Merck (Darmstadt, Germany). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and the penicillin–streptomycin mix were purchased from Gibco (Carlsbad, CA, USA). Normal melting point and low melting point agars (CAS: 9012-36-6) were obtained from Invitrogen (Carlsbad, CA, USA), and GelRed™ (CAS: 7732-18-5) was purchased from Biotium (Hayward, CA, USA). All other chemicals were analytical grade products with the highest purity available.

The erythrosine B and doxorubicin concentrations were selected using trypan blue dye exclusion at the moment of the harvest. The highest concentration at which cell viability was ≥80% was identified, and the other concentrations were based on this concentration.

Erythrosine B and doxorubicin were dissolved in DMSO at 50 mg mL\(^{-1}\) and phosphate buffer solution (PBS, pH 7.4) at 7 mg mL\(^{-1}\), respectively, just before use. The highest DMSO concentration applied to the cell cultures was 0.7%.

2.2. Cell line

A human derived liver cell line (HepG2, HB-8065, hepatocellular carcinoma) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM containing 10% heat-inactivated FBS and 1% antibiotic mix (penicillin/streptomycin). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air in an incubator (Forma Series II, Thermo Electron Corporation, USA).

2.3. Comet assay

The alkaline single-cell gel electrophoresis assay (comet assay) was performed according to Singh et al. (1988) and Tice et al. (2000), with minor modifications. Briefly, 2 × 10\(^5\) HepG2 cells were seeded in a 24-well plate for 24 h. The cells were then treated with erythrosine B at 0.1, 0.2, 2.0, 10.0, 25.0, 50.0 or 70.0 μg mL\(^{-1}\) (final concentration of 4 h) or vehicle control (0.7% DMSO) and positive control (doxorubicin 0.3 μg mL\(^{-1}\)) treatments were also performed. The HepG2 cell suspension was mixed with 37 °C low-melting point agarose and transferred to normal-melting point agarose-coated slides. The slides were then covered with a cover slip and incubated at 4 °C for 20 min for agarose solidification. The cover slips were removed, and cells were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100) overnight at 4 °C. The slides were then incubated in electrophoresis solution (300 mM NaOH and 1 mM EDTA) for 40 min at 4 °C and then transferred to a horizontal electrophoresis unit containing the same solution. The electrophoresis conditions were 25 V (0.78 V/cm) and 300 mA/C\(_{176}\) for 20 min for agarose solidification. The slides were then washed in a neutralization buffer (0.4 M mannitol, 0.02 M Tris, pH 7.4) for an additional 28 h, harvested, treated with cold hypotonic solution (1% sodium citrate) and fixed with formaldehyde and methanol–acetic acid (3:1). The slides were stained immediately before analysis using 40 μg mL\(^{-1}\) acridine orange, and the binucleated cells with 1–4 micronuclei (MNi) were scored at 1000× magnification. Additionally, the frequency of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were evaluated using the criteria of Fenech (2007). The Nuclear Division Index (NDI) was also calculated to evaluate the altered mitotic activity and/or cytostatic effects according to the following formula (Eastmond and Tucker, 1989): NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1, M2, M3 and M4 are the number of cells with one, two, three and four nuclei and N is the number of cells assayed.

2.4. Cytokinesis-block micronucleus cytome (CBMN-Cyt) assay

The mutagenicity was evaluated as described by Fenech (2007), and the culture protocol followed Natarajan and Darroudi (1991), with modifications. A total of 5 × 10\(^3\) HepG2 cells were incubated in 25 cm\(^2\) culture flasks for 24 h and then treated with 0.1, 0.2, 2.0, 10.0, 25.0, 50.0 or 70.0 μg mL\(^{-1}\) erythrosine B (final concentration) or 0.03 μg mL\(^{-1}\) doxorubicin. After 20 h of treatment (44 h after the initiation of the culture), the cells were washed with PBS, the culture media was changed, and cytochalasin B (final concentration of 3.0 μg mL\(^{-1}\) ) was added. The cells were then incubated for an additional 28 h, harvested, treated with cold hypotonic solution (1% sodium citrate) and fixed with formaldehyde and methanolic-acetic acid (3:1). The slides were stained immediately before analysis using 40 μg mL\(^{-1}\) acridine orange, and the binucleated cells with 1–4 micronuclei (MNi) were scored at 1000× magnification. Additionally, the frequency of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were evaluated using the criteria of Fenech (2007). The Nuclear Division Index (NDI) was also calculated to evaluate the altered mitotic activity and/or cytostatic effects according to the following formula (Eastmond and Tucker, 1989): NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1, M2, M3 and M4 are the number of cells with one, two, three and four nuclei and N is the number of cells assayed.

A total of 500 cells per treatment were analyzed for the NDI calculation and 1000 binucleated cells for the MNi, NPBs and NBUDs frequencies. A total of three independent experiments were performed.

2.5. Statistical analysis

The data are expressed as the mean ± SD of three independent experiments. The results were analyzed using a one-way ANOVA and post hoc Dunnett’s test and were considered significantly different if the p values were 0.05 or less.

3. Results

The results of the genotoxic evaluation of erythrosine B are presented in Figs. 2 and 3.

The sensitivity of the in vitro assay system is demonstrated by comparing the positive control group (0.3 μg mL\(^{-1}\) doxorubicin) to the vehicle control group (0.7% DMSO), displaying significant increases in both the Tail Moment and Tail Intensity. Only the two highest concentrations of ErB tested (50.0 and 70.0 μg mL\(^{-1}\) ) were significantly different (p < 0.05) from the vehicle control group when the Tail Moment and Tail Intensity, which represent the extent of DNA damage, were analyzed.

For all of the tested concentrations, the cell viability using the trypan-blue exclusion method showed ≥80% viability.

Using the micronucleus assay, at least 35% binucleated cells were observed after all of the treatments, indicating a single division cycle after the addition of cytochalasin B. The CBMN-Cyt results (Table 1) showed increased MNi frequencies at six of the seven ErB concentrations (0.2–70.0 μg mL\(^{-1}\) ) when compared to vehicle control group. The difference of MNi frequencies between the vehicle and positive control groups demonstrates the efficiency of the assay. Furthermore, as shown in Table 1, the low NPBs and NBUDs frequencies were not significantly different from the vehicle control group. The cytokinesis-block micronucleus cytome assay also evaluates the Nuclear Division Index (NDI), which was similar for all of the control and experimental groups.

4. Discussion

Comet assay and CBMN-Cyt are often combined to evaluate the genotoxicity and mutagenicity of chemical compounds, including dyes and pigments, in vitro (An et al., 2007; Oliveira et al., 2010; Tsukey et al., 2007). This association provides the analysis of two steps of DNA damage: the comet assay detects genomic lesions that can be repaired (Gontijo et al., 2003), whereas CBMN-Cyt is a useful tool to evaluate the chromosomal aberrations that can be caused by genetic defects and/or exogenous endotoxins, in addition to being an early biomarker for carcinogenesis (Bonassi et al.,...
Furthermore, HepG2 cells are frequently used to evaluate genotoxins (Chequer et al., 2009; Ferraz et al., 2011) due to their capacity to produce phase I and phase II metabolic enzymes that can potentialize or inactivate chemical compounds, closely mimicking in vivo biotransformation (Knasmuller et al., 2004; Tsuboy et al., 2007).

ErB is an unique, FDA-approved xanthene dye that is widely used in foods, cosmetics and pharmaceuticals. In USA, ErB is allowed for current use, and is usually used in sweets and foods marketed to children such as popsicles, cake frosting and another sweetmeats. Concerns about the safety of this dye were raised by FDA following the publication of a report indicating that, under experimental conditions, ErB at high dose levels (4% in the diet) can have an affect on the level of circulating thyroid hormones in rats, thus carrying to an increase in the incidence of thyroid tumours. The response of the US FDA in 1990 was to remove the ErB lakes (salts), but not Erythrosine, of all foods, drugs and cosmetics, in addition to remove ErB in cosmetics and externally applied drugs (EFSA, 2011).

However, ErB consumption exceeds its ADI by 2- to 6-fold in some population (Dixit et al., 2011) and some studies describe several toxic side effects: studied since the 1980’s, ErB has already been proven to be mutagenic by the Bacillus subtilis multigene sporulation assay (Lakdawalla and Netrawali, 1988a) and nonmutagenic by Ames/Salmonella test (Lakdawalla and Netrawali, 1988b). Other studies demonstrated ErB toxicity by different in vivo and in vitro assays: Furumiya and Mizutani (2008) reported CYP3A4 (major phase I drug-metabolizing enzyme) and P-glycoprotein (major transporter of synthetic food dyes) inhibition by ErB and other food dyes. In addition, ErB inhibits both TNF–R–TNFα and CD40–CD154 protein–protein interactions, which are responsible for tumor necrosis and effective immune responses, respectively (Ganesan et al., 2011). In vivo studies of ErB in rats demonstrated toxic effects on spermatogenesis (Abdel Aziz et al., 1997) and pituitary perturbations that may promote tumor formation due to the high iodine content (Jennings et al., 1990a). Furthermore, DNA damage was verified in rat glandular stomach, colon and urinary bladder tissues just 3 h after a single high dose (200 mg kg\(^{-1}\)) (Sasaki et al., 2002).

ErB (330 \(\mu\)M = 275.84 \(\mu\)g mL\(^{-1}\)) induced chromosome aberrations in Syrian Hamster Embryo (SHE) cells in the presence of metabolic activation. The percentage of cells with polyploidy or endoreduplication was enhanced by ErB also in the presence of exogenous metabolic activation, suggesting that this food dye could be potentially genotoxic to mammalian cells (Hagiwara et al., 2006).
Mekkawy et al. (2000) treated male rats with ErB (with 0.08 and 0.4 g kg$^{-1}$ supplemented diet) for 30 days. Changes in mutagenic activities were monitored by measuring chromosomal aberrations of rat bone marrow, nucleic acids and total protein concentrations of rat liver and brain. This study found that ErB induced chromosomal aberrations. Biochemical assays indicated a significant increase in the total protein concentration and nucleic acids in the rat brain and liver by both doses. Results indicated that both ErB doses exert mutagenic activity, being the highest dose more effective than the lower one.

Within this context, the present investigation used HepG2 cells that are a human derived liver cell line to evaluate the genotoxicity and mutagenicity of different ErB concentrations. The highest dose used in these experiments ($70.0 \mu g mL^{-1}$) was defined by the trypan-blue exclusion method. The HepG2 cells were treated with 0.1–100.0 \mu g mL$^{-1}$ ErB, and trypan-blue analysis was performed after 4 and 20 h treatment (simulating the comet assay and CBMN-Cyt assay, respectively). Only concentrations ranging from 0.1 to 70.0 \mu g mL$^{-1}$ ErB showed $>80$% cell viability after both treatments and were chosen for the in vitro experiments. This study is the first to evaluate ErB using the CBMN-Cyt assay, which provides advantages when compared to other cytogenetic methods: it is possible to evaluate mutagenic and cytostatic effects in the same assay and to predict the DNA damage mechanism involved.

The in vitro results confirm both the genotoxicity and mutagenicity of this FDA-approved food dye at low and high concentrations. The Tail Moment and Tail Intensity were statistically higher in the experimental groups (50.0 and 70.0 \mu g mL$^{-1}$) than the vehicle control group, indicating the increase of DNA damage in the HepG2 cells after 4 h of exposure. In the CBMN-Cyt assay, all of the tested concentrations, with the exception of 0.1 \mu g mL$^{-1}$, were sufficient to increase the MN frequencies.

By associating the CBMN-Cyt to the comet assay after ErB treatment, these results also suggest the ErB damage mechanism involved. The CBMN-Cyt detects both aneugenic and/or clastogenic effects, while comet assay is often considered more sensitive and detects DNA single strand breaks (He et al., 2000). Then, the positive results on six tested concentrations on CBMN-Cyt and the absence of this response on 0.1, 0.2, 2.0, 10.0 and 25.0 \mu g mL$^{-1}$ treatments on comet assay could suggest an aneugenic process that resulted in permanent changes in the DNA structure. Moreover, the NPB and NBUD analyses (CBMN-Cyt assay) ensure that any observed increase is not due to gene amplification or chromosomal rearrangements.

According to Mpountoukas et al. (2010), ErB decreased the Mitotic Index of human blood lymphocytes, which can mimic in vivo metabolism, as HepG2 cells do, whereas the NDI values calculated in the present study were not altered after ErB treatment. This difference is most likely due to the tested concentrations (1 mM–100.0 \mu g mL$^{-1}$) versus maximum 70.0 \mu g mL$^{-1}$). In conclusion, although being approved for use in the food, cosmetic and pharmaceutical industries, ErB can pose health risks and should be used with caution.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgments**

The authors thank Regislaine Valeria Burin, Ph.D. and Joana D'Arc Castania Darin (FCTRP-USP) for technical assistance. Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2011/01755-0 and 2011/14115-9) supported this work.

**References**


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