Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
eduem@uem.br
Universidade Estadual de Maringá
Brasil

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Acta Scientiarum. Biological Sciences, vol. 35, núm. 4, octubre-diciembre, 2013, pp. 557-562
Universidade Estadual de Maringá
.png, Brasil

Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v35i4.18419

Cytotoxicity of erythrosine (E-127), brilliant blue (E-133) and red 40 (E-129) food dyes in a plant test system

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ABSTRACT. The objective of this work was to evaluate the cytotoxic effect of the food dyes erythrosine, brilliant blue and red 40 on the cell cycle of *Allium cepa* L. Each dye was evaluated at doses of 0.4 and 4.0 ml, at exposure times of 24 and 48 hours, in onion root tip cells. Cells and the presence of chromosomal aberrations were analyzed throughout the whole cell cycle, totaling 5,000 cells for each group of bulbs. The mitotic index was calculated and the statistical analysis was conducted through the Chi-square test ($p < 0.05$). From the obtained results, it was verified that the food additives erythrosine and brilliant blue were not cytotoxic to the cells of the test system. However, the red 40 dye, at the two evaluated doses and the two exposure times used in this bioassay have promoted a significant reduction in cell division and induced the emergence of anaphasic and telophasic bridge aberrations and micronucleated cells. Additional cytotoxicity studies should be conducted to add information to these and other previously obtained results in order to evaluate, with property, the action of these three dyes on a cellular level.

Keywords: food additive, dye toxicity, Allium cepa.

Citotoxicidade dos corantes alimentares erythrosine (E-127), azul brilhante (E-133) e red 40 (E-129) em sistema-teste vegetal

RESUMO. Este trabalho teve por objetivo avaliar o efeito citotóxico dos corantes alimentares eritrosina, azul brilhante e vermelho 40 sobre o ciclo celular de *Allium cepa* L. Cada corante foi avaliado nas doses de 0,4 e 4,0mL, nos tempos de exposição de 24 e 48h, em células meristemáticas de raízes de cebolas. Foram analisadas células em todo o ciclo celular e a presença de aberrações cromossômicas, totalizando 5.000 células para cada grupo de bulbos. Calculou-se o índice mitótico e a análise estatística foi feita por meio do teste Qui-quadrado ($p < 0,05$). A partir dos resultados obtidos, verificou-se que os aditivos alimentares eritrosina e azul brilhante não foram citotóxicos às células do sistema-teste em questão. Já o corante vermelho 40, nas duas doses avaliadas e nos dois tempos de exposição estipulados para este bioensaio, promoveu redução significativa da divisão celular e induziu o aparecimento de aberrações dos tipos ponte anafásica, ponte telofásica e célula micronucleada. Estudos adicionais de citotoxicidade devem ser conduzidos para se somar a estes para assim avaliar, com propriedade, a ação destes três corantes em nível celular.

Palavras-chave: aditivo alimentar, toxicidade de corante, *Allium cepa*.

Introduction

In recent decades, the replacement of fresh by processed foods, rich in artificial food dyes, is gradually contributing to the impoverishment of the diet. These dyes consist of a class of food additives that provide, intensify or restore the color of a food without the purpose of nourishment (CONSTANT et al., 2007). They are considered a controversial progress of the food industry, because, from a health point of view, these compounds are not recommended (CHEESEMAN, 2012).

Among synthetic dyes most used by the food market are erythrosine, included in the class of xanthene dyes, and brilliant blue, classified as a triphenylmethane dye (BRASIL, 2005). The erythrosine is used in coloring drinks, cookies, sweets, bakery products, meat products, chewing gums and ice creams (MITTAL et al., 2006). According to Spellmeier and Stulp (2009), this dye potentially causes allergic reactions in the eyes and skin, severe headaches and nausea. The brilliant blue is used in coloring dairy products, sweets in general and pharmaceutical and cosmetic products (BESSONOV et al., 2011). This additive can provoke hyperactivity, allergic reactions, eczema and asthma, mainly in children (QUEIROZ; STEFANELLI, 2011). However, despite these adverse reactions, Polônio and Peres (2009) reported that the

use of these two dye compounds is controversial throughout the world where, for instance, in Canada and in England they are liberated to color only some foods and in Brazil their use is freely liberated.

Another additive widely used by the industry is the red 40, a dye belonging to the azo class. In Brazil it is extensively used in coloring cereals, candies, dairy products, jellies, ice cream, stuffings, liqueurs, powdered juices, soft drinks and yogurts (PAN et al., 2011). In general, this class of dyes is controversial in relation to its toxic activity and arouses the interest of toxicologists and allergists, being suggested as responsible for provoking various immunological reactions, causing from hives to asthma. It is considered that one out of 10 thousand people present adverse reactions to azo dyes (WHO, 2005). In function of that, in many countries, as in England and Japan, they are banned; however in others, as in Brazil and in the United States, the use of these additives is allowed (MORRISON et al., 2011).

Worldwide, food dye use control is based on the Acceptable Daily Intake (ADI) following results of international research and the recommendations of the Codex Committee on Food Additives and Contaminants (CCFAC) (BESSONOV et al., 2011). In Brazil, permission for use and establishment of maximum acceptable levels of food additives is responsibility of Agência Nacional de Vigilância Sanitária (ANVISA) (Brazilian National Agency of Sanitary Surveillance) and Health Ministry, which conducts this activity through the Permanent Committee on Food Additives (CPAA) (BRASIL, 2005; FAVERO et al., 2011).

Nevertheless, in spite of the control required by these regulatory agencies, the use of dyes in foods still raises a series of doubts as to their cytotoxicity, because in literature, there is a shortage of works evaluating the toxicity of these compounds (FENG et al., 2012), especially erythrosine, brilliant blue and red 40 dyes. According to Rutkunas et al. (2010), the action of these compounds, on a cellular level, should be evaluated in various test organisms, as in mammals, plants, insects and *in vitro* cell cultures, in order to measure accurately the real toxicity of these food additives.

Bioassays with plants have been considered quite sensitive and simple in monitoring the cytotoxic effects of chemical compounds (USEPA) (IGANCI et al., 2006) and the *Allium cepa* (onion) has been indicated as an efficient plant-test organism for cytotoxicity evaluation (FISKESJÖ, 1985) due to, among other characteristics, its kinetic properties of proliferation, for possessing large chromosomes and in reduced number ($2n = 16$). These characteristics facilitate the analysis to check for alterations in the cell division index (mitotic index), as in the increase or reduction in proliferation of cells of meristem tissues exposed to chemical compounds of interest (TABREZ et al., 2011), and demonstrate satisfactory similarity to the results obtained with other bioassays such as those conducted with animals and in cell cultures (ARUNG et al., 2011).

Therefore, due to the wide use of erythrosine, brilliant blue and red 40 food dyes in the food industry, and their adverse reactions, the need for additional studies and the improved researcher commitment towards evaluating the action of these dyes on a cellular level, and considering the *Allium cepa* system as an appropriate bioassay to assess the cytotoxicity of chemical compounds, this study aimed to analyze the cytotoxic effect of these three food dyes in *Allium cepa* root meristem cells.

Material and methods

This work was developed at the Plant and Animal Cytogenetics Laboratory of the Senator Helvídio Nunes de Barros Campus, from October 2010 to June 2011.

Flasks containing 10ml of erythrosine, brilliant blue and red 40 (Mix Coralin, Brazil, São Paulo) dyes were obtained at a bread-making products store located in the municipal district of Picos, Piauí State. We decided to evaluate each dye in the form in which it is used by the population. The dye solution of each flask was made up of 90% of the dye of interest and 10% of other sugars.

Two doses for each dye were defined based on the recommendations on the label of the flasks, which was 10 mL dye per 2 kg mass. Thus, the volume of 10 mL was extrapolated to the weight of each onion bulb that, on average, was 0.08 kg. The first dose established for each of the dyes under study was 0.4 mL and the second, 10 times higher than the first, 4 mL.

Onions (*Allium cepa* L.) were allowed to root in flasks with distilled water, at room temperature, approximately 25ºC, and aerated, until obtaining roots with about 1.0 cm length. For analysis of each dose, an experimental group was established containing five onion bulbs. As such, analyses were made up of two groups, one of 0.4 and one of 4 ml, for each color additive under study. Before putting the roots of the bulbs in contact with their respective doses, some roots were collected and fixed to serve as control. Afterwards, some roots were removed and fixed. After fixation, remaining roots of each bulb were again placed in their respective doses, remaining there for additional 24 hours, and ending after 48 hours exposure time. After this, roots were again collected and fixed.

The fixation of roots was performed in Carnoy 3:1 (ethanol: acetic acid), for approximately 6 hours. After the fixation, roots were subjected to hydrolysis in HCL for 8 minutes and then stained with 2% Orcein-Acetic (2 g 45 mL^{-1}). Soon afterwards, cytology slides were prepared according to Guerra and Souza protocol (2002). For each bulb, at least four slides were mounted (two roots was used for each slide) and the analysis was conducted in a 40X ZEISS 2000 optical microscope, where the number of dividing cells and the presence of cellular aberrations were observed. One-thousand-cells of each bulb were analyzed, totaling 5,000 cells per experimental group. The mitotic index was calculated by the number of dividing cells, divided by the total analyzed cells. The statistical analysis of the data was carried out by the Chi-square test at 5% of significance using the software BioEstat (AYRES et al., 2007).

Results

Table 1 lists the number of cells in interphase and in different phases of cell division and the mitotic index values obtained from *Allium cepa* root meristem cells treated with water (CO) and with erythrosine, brilliant blue and red 40 for 24 and 48 hours (ET 24h and ET 48h). In the description of the Table 1, significant χ^2 values obtained are also shown.

Table 1. Total number of cells analyzed in root tips of *Allium* cepa treated with water (control) and 0.4 and 4.0 mL erythrosine, brilliant blue or red 40 food dyes at exposure times of 24 and 48h. For each treatment were analyzed 5,000 cells.

Dye/Dose	Treatment	Cells in Interphase	Cells in Division	Mitotic Index (%)
Erythrosine	CO	4380	620	12.4 a
0.4 mL	ET 24	4265	735	14.7 a
	ET 48	4245	755	15.1 a
Erythrosine	CO	4055	945	18.9 a
4.0 mL	ET 24	4015	985	19.7 a
	ET 48	4005	995	19.9 a
Brilliant Blue	CO	4475	525	10.5 a
0.4 mL	ET 24	4410	590	11.8 a
	ET 48	4345	655	13.1 a
Brilliant Blue	CO	5615	615	12.3 a
4.0 mL	ET 24	4255	745	14.9 a
	ET 48	4205	795	15.9 a
Red 40	CO	3665	1335	26.7 a
0.4 mL	ET 24	4380	620	12.4 b
	ET 48	4790	210	4.2 c
Red 40	CO	3875	1125	22.5 a
4.0 mL	ET 24	4720	280	5.6 b
	ET 48	4830	170	3.4 b

CO – Control; ET – Exposure time. Mean values followed by the same letter are not significantly different by χ^2 test at 5%.

Dyes erythrosine and brilliant blue, at both concentrations and exposure times studied, have not significantly altered the cell division index when compared to their respective controls. However, for the red 40 dye at 0.4 mL, mitotic indexes obtained for the exposure time of 24 hours (χ^2 = 16.49) and 48 hours (χ^2 = 120.52) have significantly differed from the cell division index obtained for their controls. Also for this same dose, values of mitotic indices obtained for the two exposure times have statistically differed to each other (χ^2 = 16.0). Even though IM values of CO have been high, the inhibition of IM for the treatments was high, especially in the longer exposure time.

At the dose of 4.0 mL mitotic indices obtained for 24 hour (χ^2 = 51,0) and 48 hours (χ^2 = 107.3) exposure times have been significantly distinct in relation to the mitotic index of their respective controls.

Table 2 presents the number of cells with bridges in anaphase-telophase and micronucleated cells, and the total chromosomal aberrations present in the cells of the root meristem tissue of *Allium cepa* treated with water and with the red 40 food dye, at the exposure times of 24 and 48 hours.

Table 2. Number of micronucleated cells and cells with bridges in anaphase-telophase, and total aberrant cells treated with 0.4 and 4.0 mL of the Red 40 food dye at exposure times of 24 and 48 hours.

Dye/Dose	Treatment	Bridges in Anaphase-Telophase	Micronucleated Cells	Total Aberrant Cells
Red 40	CO	00	00	00 a
0.4 mL	ET 24h	02	25	27 b
	ET 48h	04	21	25 b
Red 40	CO	00	00	00 a
4.0 mL	ET 24h	05	20	25 b
	ET 48h	00	25	25 b

Numbers of total aberrant cells with the same letter are not significantly different by χ^2 test at 5%.

The red 40 dye, at the two doses and two exposure times herein examined, presented a statistically significantly different number of cellular aberrations in comparison with their controls. Besides, when confronted cellular aberration values obtained between the two exposure times, at the two evaluated doses, a non-significant result was observed. The presence of chromosomal aberrations was not verified for any of the two tested doses of the erythrosine and brilliant blue dyes.

Discussion

The food industry is one of the fastest growing economic sectors in the world, generating high competitiveness among producers that are in search of meeting new consumer demands. For that, they try to produce attractive foods from the hygienic, nutritional and sensory point of view. However, aiming at becoming competitive, they increasingly use food additives, which in general are any ingredient intentionally added to foods to modify

their physical, chemical, biological or sensory characteristics, without nutritional purpose.

Insofar as food dyes are classified as additives and therefore, are only directly related to the promotion of the food appearance improvement, responsible inspection agencies should present rigorous standards for evaluation and liberation of their use, because the consumption can be associated with irreversible damages to consumer health. According to Prado and Godoy (2007), approximately 2,700 chemical food additives are available on the market. As a result, the presence of different dyes in foods justifies the interest and the permanent need for evaluating their innocuity, as well as to precisely regulate their use.

The xanthene class of food dyes is widely used in coloring foods, especially Erythrosine, being behind only the Azo dye class. The food dye erythrosine is a cyclic compound with three aromatic rings in a linear arrangement with an atom of oxygen at the center of the ring, chemically named as a disodium salt of tetraiodofluorescein (PERUSSI, 2007).

Corroborating the non-cytotoxicity results of erythrosine dye obtained in this work, Wada et al. (2004) evaluated this dye with Ames bioassay tests and verified that erythrosine was neither cytotoxic nor mutagenic. Antunes and Araújo (2000) verified the non-cytotoxic activity of erythrosine on rodent colon cells, and also observed an antimutagenic activity of this dye on lesions caused by the 7, 12-dimethylbenzene in colon of mice.

Nevertheless, Prado and Godoy (2007) reported that this additive has potential of promoting cell division alterations in rodent thyroid cells, in function of releasing a great amount of iodine in the organism of these animals. Sasaki et al. (2002) verified that the erythrosine dye, at low doses and acute treatments, was highly toxic to the stomach, colon and bladder cells of Wistar rats. Only a few and recent works were found in the scientific literature evaluating the toxicity, on a cellular level, of the Erythrosine food dye.

The Brilliant Blue dye possesses a basic structure made up of three aryl radicals, in general phenolic groups, linked to a central carbon atom. It also presents sulfonic groups that gives high solubility in water, this being one of the main attractions for the food industry (PRADO; GODOY, 2003).

Different from the non-cytotoxicity results obtained in the present work, Park et al. (2009) verified that the brilliant blue dye in association with allura red and tartrazine food dyes enhances drastic alterations in the neurogenesis of rats and mice, being highly cytotoxic. Similarly to Park and collaborators, Lau et al. (2006), observed that this dye, combined with L-glutamic has been highly toxic to neurogenesis of young rodents. However, corroborating the results obtained in this work for this dye, Prado and Godoy (2003) observed that the brilliant blue was neither cytotoxic nor mutagenic in bone marrow cells of Wistar rats.

As well as with erythrosine, there are few recent works in the scientific literature about the activity on a cellular level of the brilliant blue dye. Some studies evaluating the action of this dye was conducted in the 80's and 90's, and the results, as those obtained here, evidenced that the brilliant blue was not cytotoxic to bone marrow cells of rats and in rodent cell culture.

The Azo group dyes present a naphthalene ring bonded to a second benzene ring by an azo bond (N = N). Those rings can contain one, two or three sulfonic groups. Worldwide, this group represents the most used class of synthetic dyes by the food industry, especially the red 40 (MORRISON et al., 2011).

Recent works in the scientific literature evaluating the cytotoxicity, mutagenicity or carcinogenicity of red 40 food dye were not found. The few existing works are from the 80's. The results of these researches are contradictory, in which some showed cytotoxicity and mutagenicity, similar to the results obtained in our work, and others have evidenced no cytotoxic activity and even an antimutagenic potential.

This shortage of information on red 40-food dye becomes troubling because this additive is widely used in the food market, especially by Brazilian industries. As already mentioned, the Azo class dyes are controversial in the scientific community as to their toxicity, including on a cellular level; however almost all of the studies in this area are on tartrazine, bordeaux red and sunset yellow dyes. Therefore, the need for additional studies that evaluate more adequately the activity of this food dye is justified.

Particularly, cytotoxicity results of chemical compounds in animals may be different from those obtained in plant test systems, depending on the metabolism in the animal test system. Unfortunately, there are no studies in the literature that assessed the cytotoxicity of food dyes tested in plant systems for comparison with the results obtained in animals.

In this way if the plant metabolism is different, the *A. cepa* test system is an excellent cytotoxicity parameter, since the occurrence of abnormalities in the cell cycle of *A. cepa* has been used for a long time by the scientific community as indicative to advise the human population on the consumption of synthetic and natural medicines.

Conclusion

Further studies are necessary, with varied doses, exposure times and test organisms, to accurately evaluate the potential risks of cytotoxic agents present in the compounds of these food additives. These results will be important to advise the committees responsible for the Acceptable Daily Intake (ADI), like Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), Expert Scientific Committee Administered jointly by Food and Agriculture Organization of the United Nations (JECFA) and Agência Nacional de Vigilância Sanitária (ANVISA), on the establishment of appropriate tolerable limits in the use of these food additives.

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Received on August 27, 2012.
Accepted on February 26, 2013.