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Processed fruit juice ready to drink: screening acute toxicity at the cellular level

Erick Leal da Silva[1], Ila Monize Sousa e Sales[1], Fabelina Karollyne Silva dos Santos[1] and Ana Paula Peron[2*]

*[1]Curso de Ciências Biológicas, Universidade Federal do Piauí, Picos, Piauí, Brazil. [2]Programa de Pós-Graduação em Genética e Melhoramento, Centro de Ciências Agrárias, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, s/n., 64049-550, Teresina, Piauí, Brazil. *Author for correspondence. E-mail: anapaulaperon@ufpi.edu.br*

ABSTRACT. The present study evaluated the acute toxicity at the cellular level of processed juice ready for consumption Orange and Grape flavors, produced by five companies with significant influence on the food market of South American countries, especially in Brazil. This evaluation was performed in root meristem cells of *Allium cepa* L., at the exposure times of 24 and 48 hours, directly with marketed liquid preparations. Based on the results, it was found that fruit juices, of all companies considered, promoted significant antiproliferative effect to root meristems at the exposure time of 24 hours and resulted in at both exposure times, statistically significant number of mitotic spindle changes and chromosomal breaks. Therefore, under the study conditions, all juice samples analyzed were cytotoxic, genotoxic and mutagenic to root meristem cells. These results indicate that such beverages have relevant potential to cause cellular disorders and, thus, need to be evaluated more fully in more complex test systems, as those in rodents, and then establish specific toxicity at the cellular level of these juices and ensure the well-being of those who consume them.

Keywords: industrialized juice, cytotoxicity, genotoxicity, mutagenicity, meristem tissue.

Sucos de frutas industrializados prontos para consumo: *screening* da toxicidade aguda em nível celular

RESUMO. Objetivou-se neste trabalho avaliar a toxicidade aguda em nível celular de sucos industrializados prontos para beber, sabor laranja e uva, de cinco empresas alimentícias de reconhecida reputação no mercado de alimentos em países da América do Sul, especialmente o Brasil. Esta avaliação se deu por meio das células meristemáticas de raízes de *Allium cepa* L., nos tempos de exposição 24 e 48h, diretamente nos preparados líquidos comercializados. Com base nos resultados obtidos verificou-se que os sucos de frutas, de todas as empresas consideradas, promoveram expressivo efeito antiproliferativo aos meristemas de raízes já no tempo de exposição 24h, e ocasionaram número estatisticamente significativo de alterações de fuso mitótico e quebras cromossômicas nas células do tecido analisado em todo o tempo de análise. Portanto, nas condições de estudo estabelecidas, os sucos das empresas avaliadas foram citotóxicos, genotóxicos e mutagênicos. Estes resultados são importantes em razão de indicarem que tais alimentos têm relevante potencial em causar distúrbios celulares e, portanto, devem ser avaliados em sistemas com testes mais complexo, como os em roedores, para, dessa forma, estabelecer com propriedade a toxicidade em nível celular desses alimentos e assegurar o bem-estar daqueles que os consomem.

Palavras-chave: sucos industrializados, citotoxicidade, genotoxicidade, mutagenicidade, tecido meristemático.

Introduction

Because of rapid pace of life, the manual preparation of fruit juices has become an inconvenience to daily life. Thus, the world consumption of processed juices has tripled in the last twenty years, which strengthened the attachment of various trademarks in domestic and international market of processed fruit juices ready for consumption (Biral, Taddei, Passoni, & Palma, 2013, Longo-Silva et al., 2015). In addition to the convenience in consumption, ready juices have excellent sensory properties such as color, odor and attractive flavor, are available in various fruit flavors and sold in compact packages, which facilitates storage (Ferrarezi, Santos, & Monteiro, 2010).

However, in order to achieve the organoleptic properties currently offered, the composition of these beverages has undergone several changes over nearly two decades, among which the significant incorporation of food synthetic additives or micro

ingredients with acidifying, antioxidant, flavoring, coloring, anti-wetting, acidity regulators, foaming and sweetening actions (Ferrarezi et al., 2010, Longo-Silva et al., 2015). These changes were approved and standardized by the Ministry of Agriculture, Livestock and Supply and the National Health Surveillance Agency (Anvisa) through Ordinance 544 of November 16th, 1998 approving the technical regulations for setting of identity and quality standards of processed juices and refreshments marketed in liquid form (Brasil, 2000, Ferrarezi et al., 2010).

Meanwhile, there are studies that relate the micro ingredients to harmful effects to the health of consumers, especially to children. The consequences described include the development or enhancement of chronic allergies and changes in the functioning of the digestive tract (Gomes, Oliveira, Carvalho, Menezes, & Peron, 2013, Oliveira, Alves, Lima, Castro, & Peron, 2013, Moura, Santana, Ferreira, Sousa, & Peron, 2016). Thus, food surveillance agencies, such as the European Food Safety Authority (EFSA) and Anvisa, point out in their technical regulations the constant need for cytotoxicological studies on the acute effect of food additives in general and especially processed food containing such substances. They also emphasize that the results of these analyzes are the basis of preparation or modification of strategies of food safety agencies and, consequently, the work of professionals responsible for food and nutrition surveillance of the population (Brasil, 2007, Moura et al., 2016, Carvalho, Sales, & Peron, 2017). However, a comprehensive analysis of the scientific literature indicated the lack of studies on the toxicity of processed juices sold in liquid form.

The meristem tissue of roots of *Allium cepa* L. (onion) is regarded in the scientific community as an effective bioassay for the assessment of acute toxicity at the cellular level of chemical compounds, due to low chromosome number (2n = 16), which facilitates the detection of chromosomal abnormalities and mitotic spindle changes (Neves, Ferreira, Lima, & Peron, 2014, Bianchi, Mantovani, & Marin-Morales, 2015). This test system is internationally accepted by research agencies as an assessment tool with accurate sensitivity for initial analysis of cytotoxicity, genotoxicity and mutagenicity of the substance of interest, as the results obtained show satisfactory similarity to those observed with animal test systems and cell cultures (Türkoğlu, 2007, Herrero et al., 2011, Tabrez et al., 2011, Gomes et al., 2013, Oliveira et al., 2013, Lacerda, Malaquias, & Peron, 2014, Campos-Ventura & Marin-Morales, 2016, Moura et al., 2016, Santana et al., 2016, Campus-Pereira, Gonçalves, Hara, & Marin-Morales, 2016).

As described by Zilifdar et al. (2014) and Valavanidis, Vlachogianni, Fiotakis, and Loridas (2013), cytotoxic, genotoxic and mutagenic substances can damage vital cellular mechanisms, such as gene duplication and transcription, changing dramatically the cell division of tissue through cellular aberrations, such as mitotic spindle changes and chromosome breaks, which can trigger and/or potentiate cancerous processes. According to Zaineddin et al. (2012), the development of the most common types of cancer results from the interaction between endogenous and environmental factors, remarkably the diet, especially when made of processed foods in excess.

Based on the discussed context, the goal of this study was to evaluate cytotoxicity, genotoxicity and mutagenicity of processed liquid preparations ready for consumption, Orange and Grape flavors, of five relevant food companies in the Brazilian market, through the meristematic cells of *A. cepa* roots. The Orange and Grape juices were selected for analysis based on studies conducted by Ferrarezi et al. (2010) and Pontes, Santiago, Szabo, Toledo, and Gollucke (2010), which point them as the most appreciated by the population.

Material and methods

Study samples

Samples of processed juices ready for consumption, Orange and Grape flavors, from five different food companies - referred to in this study as A, B, C, D and E- were acquired in the retail market in the city of Picos, state of Piauí, Brazil. We were careful to check whether the samples were within the shelf life and the packaging were intact. Analyses of toxicity were carried out directly with the marketed solutions.

Obtaining root meristem cells of *A. cepa* and cytogenetic analysis

Onion bulbs were placed in aerated bottles with distilled water at room temperature (± 27°C) to obtain 2.0 cm long roots. For analysis of each treatment group, we established an experimental group with five onion bulbs. Before placing the roots in contact with their respective samples of juices (treatments), some roots were collected and fixed to serve as control of the bulb it self. The remaining roots were placed in their respective solutions for 24 hours, a procedure called exposure time of 24 hours.

After 24 hours, some roots were collected and fixed. The remaining roots of each bulb were returned to their respective solutions, where they remained for more 24 hours, which is termed exposure time of 48 hours. Subsequently, again, roots were collected and fixed. The exposure times 24 and 48 hours were chosen to evaluate the effect of treatments on more than one cell cycle. Roots were fixed in Carnoy 3: 1 (ethanol: acetic acid) for 24 hours. We collected on average three roots per bulb in each collection. In all exposure times considered, the bottles with the treatments under study remained under gentle and constant stirring. This procedure was carried out to prevent the precipitation of solutions.

Slide preparation and analysis, and statistical analysis

On average, 3 slides per bulb were prepared following the protocol proposed by Guerra and Souza (2002), and analyzed under an optical microscope at 400x magnification. For each onion bulb, we analyzed 1,000 cells, totaling 5,000 cells for each control, exposure time 24 and 48 hours of each treatment group. Cells were observed in interphase, prophase, metaphase, anaphase and telophase. For calculation of the mitotic index (MI), we used the following equation: (total number of cells in mitosis ÷ total number of cells analyzed) x 100.

It was also assessed the action of juice samples by the frequency of micronuclei, colchicine metaphase, anaphase and telophase bridges, gene amplifications, cells with adhesions, nuclear buds and multipolar anaphases. For statistical analysis, we used the chi-square test (χ^2) at a probability level < 0.05.

Results and discussion

Considering the results in Table 1, it was observed that the Orange and Grape juices of the company A significantly reduced the cell division index at the 24 hours exposure time compared to the mitotic index observed for its respective control. This result is more pronounced at the 48 hours exposure time, in which the cell division index was statistically lower than those observed for the respective controls and 24 hours exposure time. In turn, Orange and Grape juices of the companies B, C, D and E caused a significant reduction in cell division of the meristem tissue, in both exposure times considered, compared to the mitotic index observed for the respective controls. However, there were no significant differences in the cell division index between the respective exposure times of 24 and 48 hours.

These results show that all juice samples tested, under the conditions established, were cytotoxic to root meristem cells of *Allium cepa*, because they have caused marked anti proliferative effect to root meristems. According to Gomes et al. (2013), Moura et al. (2016) and Carvalho et al. (2017), the reduction in mitotic index caused by chemical compounds in tissues without any mutation and/or cellular change is harmful to the organism in which they occur, as it does not allow or limit the replacement of cells, altering the production of proteins and therefore resulting in malfunction of the organ where it is located.

Table 1. Number of cells observed in each phase of the cell cycle of the root meristem tissue of *Allium cepa* treated with processed juices ready for consumption, orange and grape flavors, of the food companies A, B, C, D and E, at the exposure times of 24 and 48 hours.

Orange Processed Juice								
Company	ET	TCII	P	M	A	T	TCD	MI (%)
A	CO	4136	319	146	267	132	864	17.3[a]
	24 hours	4645	130	84	72	69	355	7.1[b]
	48 hours	4849	56	30	41	24	151	3.0[c]
B	CO	3971	481	207	179	162	1029	20.6[a]
	24 hours	4744	177	23	18	38	256	5.1[b]
	48 hours	4846	93	29	16	36	174	3.5[b]
C	CO	4322	232	158	136	152	678	13.6[a]
	24 hours	4806	101	54	12	17	194	3.9[b]
	48 hours	4828	58	52	12	10	172	3.4[b]
D	CO	4201	251	119	246	183	799	15.9[a]
	24 hours	4745	98	71	50	36	255	5.1[b]
	48 hours	4823	83	34	31	29	177	3.5[b]
E	CO	4076	302	292	187	143	924	18.4[a]
	24 hours	4633	99	97	82	89	367	7.3[b]
	48 hours	4678	83	79	79	81	322	6.4[b]
Grape Processed Juice								
Company	ET	TCII	P	M	A	T	TCD	MI (%)
A	CO	4282	534	80	43	61	718	14.4[a]
	24 hours	4685	274	12	09	20	315	6.3[b]
	48 hours	4847	96	20	06	31	153	3.1[c]
B	CO	4298	499	93	47	63	702	14.4[a]
	24 hours	4159	71	21	07	42	141	2.8[b]
	48 hours	2971	71	11	00	47	129	2.6[b]
C	CO	4422	232	158	136	52	578	11.6[a]
	24 hours	4806	101	54	12	17	194	3.9[b]
	48 hours	4838	78	60	02	22	162	3.2[b]
D	CO	4305	189	191	117	198	695	13.9[a]
	24 hours	4704	44	57	13	22	196	3.9[b]
	48 hours	4817	89	71	17	06	183	3.7[b]
E	CO	4399	178	176	176	71	601	12.0[a]
	24 hours	4750	78	86	53	33	250	5.0[b]
	48 hours	4824	69	45	41	21	176	3.5[b]

TCII – Total number of cells in interphase and undifferentiated cells; ET – Exposure Time; CO – Control; MI – Mitotic Index; TCD – Total number of dividing cells. MI values followed by different letters within the same treatment are significantly different at 5% by χ^2 test.

With respect to the results listed in Table 2, the orange and grape juices of the five food companies, at both exposure times considered, induced significant formation of mitotic spindle changes, represented in this study by colchicine metaphase and anaphase and telophase bridges, proving to be genotoxic, and chromosome breaks, characterized by the formation of micronuclei.

Mitotic spindle changes, at significant frequency, provoke nuclear instability by inducing structural chromosome damage, giving rise to acentric fragments, which therefore form micronuclei at the end of cell division. The significant presence of micro nucleated cells, as observed herein with ready juices, classifies substances, compounds or solutions tested as mutagenic (Corcuera et al., 2015). This condition with the liquid preparations indicates that they should be evaluated in animal test systems, since, according to Queiroz, Matias, Cunha and Schwarz (2013), the occurrence of cell changes, though not considered a carcinogenicity measurement, are often associated with the appearance of cancer, as there is a positive correlation between increased mitotic spindle aberrations, as well as micro nucleated cells, and detection of neoplasms.

Table 2. Cell changes observed in root meristem cells of *Allium cepa* treated with water and with processed juice ready for consumption, orange and grape flavors, of the food companies A, B, C, D and E, at the exposure times of 24 and 48 hours.

Company	ET	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronuclei	TAC
	CO	00	00	00	01	01^a
A	24 hours	32	11	09	37	89^b
	48 hours	13	17	11	32	73^b
	CO	00	01	00	00	01^a
B	24 hours	12	02	03	25	42^b
	48 hours	08	17	07	21	53^b
	CO	00	01	00	00	01^a
C	24 hours	09	04	04	32	49^b
	48 hours	02	01	08	30	41^b
	CO	01	00	00	00	01^a
D	24 hours	23	17	02	44	86^b
	48 hours	19	19	00	23	71^b
	CO	01	00	00	00	01^a
E	24 hours	24	07	03	42	76^b
	48 hours	09	09	11	44	73^b
Company	ET	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronuclei	TAC
	CO	01	00	00	00	01^a
A	24 hours	19	13	13	32	77^b
	48 hours	17	13	19	22	71^b
	CO	01	00	00	00	01^a
B	24 hours	22	09	09	32	72^b
	48 hours	13	13	04	40	70^b
	CO	01	00	00	00	01^a
C	24 hours	04	13	09	22	48^b
	48 hours	17	01	09	24	51^b
	CO	01	00	00	00	01^a
D	24 hours	23	19	03	24	69^b
	48 hours	11	27	00	29	67^b
	CO	01	00	00	00	01^a
E	24 hours	14	09	13	33	69^b
	48 hours	03	19	02	41	65^b

ET – Exposure Time; CO – Control; TAC – Total Cellular Alterations. MI values followed by different letters within the same treatment are significantly different at 5% by χ^2 test.

Also, the frequency of cell changes observed in Table 2 confirms the results of reduced cell division in Table 1. Aissa et al. (2012) report that metaphases with misalignment of chromosomes on the equatorial plate or colchicine metaphase, as well as delayed chromosomes in anaphase and/or telophase, or anaphase and telophase bridges, result in formation of cells with different chromosome numbers, and chromosome structural changes. Whereas the principle of the cell cycle is the formation of identical cells, cells with discrepant variations in structure and in chromosome number tend to be eliminated in tissues with normal functioning.

As mentioned above, there is no toxicological evaluation in the scientific literature to date, at the cellular level regarding processed juices sold in liquid form. However, there are cytotoxicity assessments of some of the chemical constituents of the food additive classes in the formulation of these beverages, as described in the technical document that regulates these liquid preparations (Brasil, 2007). Nevertheless, it is very important to mention that the chemical composition of processed fruit juices ready for consumption, such as those from the five companies evaluated here in, is allowed and guaranteed by Brazilian law (Brasil, 2007, Ferrarezi et al., 2010; Pontes et al., 2010).

Artificial food dyes found in orange juice ready for consumption are the Twilight Yellow and Tartrazine. As for the grape, one of the dyes used is the Red 40. The dyes Sunset Yellow, Tartrazine and Red 40 are classified as azo food additives, because they have an azo group in their formulation, which is a nitrous derivative property to produce aromatic amine and sulfanilic acid (Sardi et al., 2010). These color additives have the potential to alter the turnover of cells during interphase and the regenerative hyperplasia process, contributing significantly to the development of digestive tract cancers in rodents (Polônio & Peres, 2009, Mpountoukas et al., 2010). These three azo dyes were also significantly cytotoxic, mutagenic and genotoxic to meristematic cells of *Allium cepa* roots (Gomes et al., 2013).

The diluents present in taste and flavor additives include benzoic alcohol and diacetyl (2,3-butadione), compounds which promote significant changes in the mitotic spindle and cell division of human peripheral blood cells (Demir, Kocaoglu, & Kaya, 2010) and significant damage to the chromosome 11 locus of rodents, causing loss of expression of essential genes controlling cell division (Whittaker, Clarke, San, Begley, & Dunkel, 2008), respectively. Furthermore, More, Raza, and Vince (2012) found that the diluent diacetyl had the potential to replace thymine with guanine in euchromatin regions, resulting in the disruption of hydrogen and disulfide bonds in the tertiary

structure of enzymes important for mitosis. Among the preservatives stand out potassium benzoate, sodium benzoate, potassium nitrate, cytotoxic and genotoxic effects to normal cells of human peripheral blood (Mpountoukas et al., 2010, Zequin, Yüzbaşioğlu, Unal, Yilmaz, & Aksoy, 2011), boric acid, citric acid, potassium citrate and sodium citrate, cytotoxic and genotoxic to root meristem cells of *A. cepa* (Türkoğlu, 2007).

The sweeteners used in liquid preparations ready to drink comprise aspartame, sodium cyclamate, acesulfame potassium and sodium saccharin, with the exception of juices ready for consumption of Ambev, which have no sweeteners in their chemical composition, according to the site of presentation of the products. Van Eyk (2015) found that, through the cell lines Caco-2 (colon cells), HT-29 (colon cells) and HEK-293 (kidney cells), these sweeteners were cytotoxic, genotoxic and mutagenic to the studied cells. Corroborating the results of these researchers, Sasaki et al. (2002), using the comet assay, observed that sodium saccharin and sodium cyclamate were genotoxic and mutagenic to rodent colonic cells.

Conclusion

All juice samples analyzed were cytotoxic, genotoxic and mutagenic to root meristematic cells of *A. cepa*.

The results of this study, combined with the toxicity data at the cellular level of some of the food additives present in the basic formulation of juices ready to drink, signal the need for more detailed assessments, intest systems with higher complexity, to then safely establish the toxicity at the cellular level of these foods.

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