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Toxicity at the cellular level of artificial synthetic flavorings

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ABSTRACT. The goal of the present study was to evaluate the cytotoxicity and genotoxicity of artificial synthetic flavoring agents cookie and tutti-frutti. To this end, root meristem cells of *Allium cepa* L. were exposed to these substances in exposure times of 24 and 48 hour using individual doses of 0.3; 0.6 and 0.9 mL and doses combined as follows: 0.3 mL + 0.3 mL; 0.6 mL and 0.9 mL + 0.6 mL + 0.9 mL. After applying the treatments, root meristems were fixed, hydrolyzed, stained and analyzed a total of 5,000 cells using an optical microscope to evaluate each dose and combined treatment. All three doses of cookie flavoring and combined treatments significantly inhibited cell division of the tissue studied. Doses of tutti-frutti caused no change in cell division rate. In addition, doses of both flavorings and treatments combining these solutions induced cell aberrations in a significant number of cells to the *A. cepa* system. Therefore, under these analytical conditions, cookie flavoring and combined doses were cytotoxic and genotoxic, and tutti-frutti flavoring, although non-cytotoxic, demonstrated genotoxic action.

Keywords: flavorings, cell division, cell aberrations, cytotoxicity, genotoxicity.

Toxicidade em nível celular de aromatizantes sintéticos artificiais

RESUMO. Objetivou-se neste trabalho avaliar a citotoxicidade e a genotoxicidade de aditivos aromatizantes sintéticos artificiais de biscoito e tutti-frutti. Essa avaliação se deu por meio das células meristemáticas de raízes de *Allium cepa* L., nos tempos de exposição de 24 e 48 horas, em que estas substâncias foram analisadas individualmente, nas doses de 0,3; 0,6 e 0,9 mL, e associadas entre si, da seguinte forma: 0,3 mL + 0,3 mL; 0,6 mL + 0,6 mL e 0,9 mL + 0,9 mL. Os meristemas de raízes foram fixados, hidrolisados e corados, e em seguida analisados em microscópio óptico, no qual se avaliou para cada dose e tratamento associado um total de 5.000 células. A partir dos resultados obtidos verificou-se que as três doses do aromatizante de biscoito e os tratamentos em associação inibiram significativamente a divisão celular dos tecidos em estudo. Já as doses de tutti-frutti não alteraram o índice de divisão celular das raízes em questão. Verificou-se também que as doses dos dois aromatizantes e os tratamentos em associação, induziram aberrações celulares em número significativo. Portanto, nestas condições de análise, o aditivo de biscoito e as doses associadas foram citotóxicos e genotóxicos, e o de tutti-frutti, apresentou apenas ação genotóxica.

Palavras-chave: aromatizantes alimentares, divisão celular, aberrações celulares, citotoxicidade, genotoxicidade.

Introduction

In food industry, food additives or microingredients have become essential to enhance sensory properties and extend the shelf life of processed foods. The most important additives include flavorings, substances with aromatic and taste properties able to confer and/or intensify the aroma and the taste of foodstuffs without nutritional purposes (Brasil, 2007; Koca, Erbay, & Kaymak-Ertelain, 2015). Classified as natural, synthetic nature-identical and synthetic artificial, aroma and taste microingredients have a complex formulation comprising a variety of chemical compounds, such as diluents, antioxidants, defoamers, preservatives, emulsifiers, stabilizers, acidity regulators, flavor enhancers, antiwetting agents, anti-caking agents, dyes, and extraction and processing solvents. Worldwide, flavoring substances are regulated and authorized for use by the Food and Agriculture Organization (FAO) (Xu et al., 2013), and in Brazil by the National Sanitary Surveillance Agency (ANVISA) by Resolution RDC 2 of January 15th, 2007 (Brasil, 2007).

Although conferring essential organoleptic properties to processed foods, flavoring additives are a controversial advancement in the area of food science and technology by many health professionals

(Konishi, Hayashi, & Fukushima, 2011). Experts report that these ingredients contribute significantly to the impoverishment of the diet, cause severe disturbances in the functioning of the digestive tract and trigger allergic and narcotic reactions in the body, especially in children and elderly individuals (Konishi et al., 2011; Oliveira, Alves, Lima, Castro, & Peron, 2013). Also, to date, food surveillance agencies have not established the Acceptable Daily Intake (ADI) to ensure the safe use of these substances (Zeguin, Yüzbaşioğlu, Unal, Yilmaz, & Aksoy, 2011; More, Raza, & Vince, 2012). Because of such considerations, food safety experts emphasize the urgent need for studies on flavoring toxicity assessment, focusing on cytotoxicity, genotoxicity and mutagenicity (Brasil, 1999; Brasil, 2007; Konishi et al., 2011; Gomes, Oliveira, Carvalho, & Menezes, 2013; Xu et al., 2013). However, there is no research in the scientific literature assessing the toxicity at the cellular level of aroma and flavor additives.

Cytotoxic and/or genotoxic compounds have the potential to change vital cellular mechanisms, such as duplication and transcription, and promote mitotic spindle changes and chromosomal breaks. These changes can significantly impair cell division of the tissue or organ affected and initiate and/or potentiate cancerous processes (Valavanidis, Vlachogranni, Fiotakis, & Lionidas, 2013; Zilifdar, Alpes-Hayta, Yilmaz, Kaplan-Orzen, & Aylogn, 2014). According to Zaineddin et al. (2012), the development of the most common types of cancer results from the interaction between endogenous and environmental factors, especially the diet, especially when it consists of excess of processed foods. Bendino, Populine and Cerqueira (2012) and Louzada, et al. (2015) report that over 40% of various cancers are initiated or exacerbated due to inadequate diets, rich in food additives.

Root meristem cells of *Allium cepa* L. (onion) are effective bioassays for the initial screening of acute toxicity at the cellular level (Herrero et al., 2012; Lacerda, Malaquias, & Peron, 2014). This test organism has excellent kinetic properties of proliferation, large chromosomes in reduced number (2n = 16), which facilitates the detection of chromosomal aberrations and abnormalities in the mitotic spindle (Cardoso, Dantas, Sousa, & Peron, 2014). It also allows the verification of changes in cell division or mitotic index when exposed to chemical compounds with potential cytotoxic action. Furthermore, this test system has, in most cases, satisfactory similarity to the results obtained with other test systems (Tabrez et al., 2011). For instance, the studies carried out by Gomes et al. (2013) and Oliveira et al. (2013) evaluated, in root meristem cells of *A. cepa*, cytotoxic and geotoxic potential of artificial synthetic food dyes and obtained results similar to those observed in animal test systems and in cell cultures.

Based on this context, this study evaluated, in root meristem cells of *A. cepa*, individually and associated with each other, the cytotoxicity and genotoxicity of two artificial synthetic flavorings, tutti-frutti, found in candy, chewing gum, gum, ice cream, jelly and soft drinks, and cookie, found in cake dough and industrialized cookies. These additives are also commonly found in combination in cupcakes and processed cakes.

Material and methods

Obtaining food flavorings, setting and analysis of the doses

Artificial synthetic aroma and flavor additives, tutti-frutti and cookies, were obtained from a manufacturing industry of food microingredients in the city of Recife, state of Pernambuco, Brazil, specialized in national and international marketing of synthetic food additives.

The label of both additives suggested the use of 3 mL of flavoring for 1.0 kg of mass. Onion bulbs selected for this study weighed on average 200 g. Thus, proportionally to that recommended, it was initially set for analysis the dose of 0.6 mL. Then, we defined two doses, 0.3 and 0.9 mL. We also assessed the combination of the two flavorings, as follows: for each tutti-frutti flavoring dose set it was added the same dose of the cookie flavoring.

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

Onion bulbs were allowed to root in bottles with aerated distilled water at room temperature (± 27°C) until obtaining 2.0 cm long roots. For analysis of each dose and combined doses (combination treatment), we set up an experimental group with five onion bulbs. Before placing the roots in contact with their respective treatments, some roots were collected and fixed to serve as control of the bulb itself. Then, the remaining roots were returned to their respective solutions for 24 hour, a procedure called 24 hour exposure time (ET 24 hour).

After 24 hours, some roots were taken and fixed. Next, the remaining roots of each onion roots were returned to their respective doses or treatments, where they remained for more 24 hours, which was called as 48 hour exposure time (ET 48 hour). Thereafter, roots again were collected and fixed. Roots were fixed in Carnoy 3: 1 (ethanol: acetic

acid) for 24 hour. Three roots per bulb were taken in each collection.

Preparation and reading of slides and statistical analysis

Slides, on average, 03 per bulb, were prepared following the protocol proposed by Guerra and Souza (2002), and analyzed by light microscopy at 40x magnification. For each onion bulb, we examined 1,000 cells, totaling 5,000 cells for the control, ET 24 hour and ET 48 hour in each treatment group. Cells were observed in interphase, prophase, metaphase, anaphase and telophase. We calculated the number of interphase and dividing cells in each control and exposure time and thus determined the index of cell division or mitotic index (MI). It was also evaluated the action of doses by means of the number of micronucleated cells, colchicine metaphases, anaphase and telophase bridges. The data were submitted to statistical Chi-square at 5%.

Results and discussion

First, it is important to mention that, according to the Technical Regulation on flavorings/aroma and flavor approved by ANVISA in 1999 and still in force, the formulation of any synthetic food flavoring is globally standardized and inspection of the composition is the responsibility of food safety agencies (Brasil, 1999, 2007). In addition, for this research, no dilution was performed to obtain the doses of flavorings. This because the flavorings, in general, have complex chemical formulation and so, the concentration and the action of compounds present in these ingredients could be changed if diluted. Furthermore, after an extensive search in sites specialized in domestic and international marketing of aroma and flavor additives, the ideal dose for consumption, recommended by different manufacturers, was almost unanimously the same as that used in this study, 3 mL tutti frutti or cookie flavoring for 1 kg of mass. The exposure times of 24 hour and 48 hour were established in order to evaluate the effect of these additives on root meristems on more than one cell cycle.

Table 1 lists the number of cells in interphase and at different stages of cell division, and the values of mitotic index obtained from root meristem cells of *A. cepa* treated with water and food flavorings cookie and tutti frutti. These additives were evaluated alone and in combination in two exposure times. The description of the results also presents the significant values of χ^2.

Table 1. Number of cells observed for each stage of the cell cycle root meristem cells of *Allium cepa* treated with water and artificial synthetic flavorings cookie and tutti-frutti, at doses of 0.3; 0.6 and 0.9 mL, assessed alone and in combination at the exposure times of 24 and 48 hours.

Cookie Flavoring								
Dose (mL)	ET	TCII	P	M	A	T	TCD	MI (%)
	CO	4090	596	155	73	86	910	18.2[a]
0.3	24h	4572	161	109	64	94	428	8.6[b]
	48h	4712	113	73	43	59	288	7.8[b]
	CO	4258	556	87	47	51	741	14.8[a]
0.6	24h	4577	249	83	46	45	423	8.5[b]
	48h	4679	136	96	48	41	321	6.4[b]
	CO	4291	482	105	50	72	709	14.2[a]
0.9	24h	4420	75	72	28	13	188	3.8[b]
	48h	4463	17	06	08	06	37	0.7[c]
Tutti-Frutti Flavoring								
Dose (mL)	ET	TCII	P	M	A	T	TCD	MI (%)
	CO	4508	279	110	54	49	492	9.8[a]
0.3	24h	4651	69	154	65	61	349	7.0[a]
	48h	4606	88	165	101	40	394	7.9[a]
	CO	4463	304	110	72	51	537	10.7[a]
0.6	24h	4684	51	147	62	56	316	6.3[a]
	48h	4664	74	128	78	56	336	6.7[a]
	CO	4208	323	137	159	73	692	11.8[a]
0.9	24h	4494	179	160	85	82	506	10.1[a]
	48h	4663	162	156	101	68	487	9.7[a]
Cookie Flavoring + Tutti-Frutti Flavoring								
Combined doses(mL)	ET	TCII	P	M	A	T	TCD	MI (%)
	CO	4165	558	153	75	69	855	17.1[a]
0.3 + 0.3	24h	4130	567	139	82	82	870	17.4[a]
	48h	4726	101	83	51	39	274	5.5[b]
	CO	4038	567	183	114	108	972	19.4[a]
0.6 + 0.6	24h	4654	104	153	51	38	346	7.0[b]
	48h	4924	34	18	17	07	76	1.5[c]
	CO	4236	410	158	94	102	764	15.3[a]
0.9 + 0.9	24h	4727	84	81	69	39	273	5.5[b]
	48h	4952	13	17	05	03	38	0.8[c]

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

Table 1 shows that mitotic indices of cells treated with the three doses of cookie flavoring were significantly lower than cell division indices obtained for the respective controls. The cell division observed for doses 0.3 and 0.6 mL in ET 48 hour, this aditive were statistically lower than those observed mitotic indices for respective ET 24 hour. Otherwise, the mitotic index registered for ET 48 hour with the dose of 0.9 mL of the cookie additive was significantly lower in relation to its specific cell division index in ET 24 hour. Thus, under the conditions analyzed, doses of the cookie flavoring proved to be cytotoxic as they significantly inhibited cell division in root meristems of the test system.

Regarding the tutti-frutti flavoring (Table 1), mitotic indices observed for the three doses in both exposure times investigated were statistically similar to the mitotic index obtained for their controls. In the same way, when comparing the cell division indices of ET 48 hour with ET 24 hour for doses of this flavoring, we found no statistically difference. Therefore, the tutti-frutti additive was not cytotoxic to cells of the test organism considered.

Still in Table 01, data for the treatments from the association between doses of cookie and tutti-frutti flavorings showed that in the treatment 0.3 mL + 0.3 mL, cell division indices for the control and ET 24 hour presented no significant differences. Nevertheless, the mitotic index obtained for this association in ET 48 hour was significantly lower than mitotic indices obtained for the respective control and ET 24 hour. For the other two associations, 0.6 mL + 0.6 mL and 0.9 mL + 0.9 mL, the observed mitotic indices for ET 24 and 48 hour were significantly different from the values of their respective controls. Likewise, when comparing the mitotic indices obtained for ET 48 hour of these two treatments with their specific ET 24 hour, there was a statistically significant reduction. Thus, treatments regarding the associations between the two flavoring additives significantly promoted antiproliferative effect of the analyzed meristems, proving to be cytotoxic.

Table 2 presents the number and types of cellular abnormalities found in meristematic cells of *A. cepa* roots treated with water and food flavorings cookie and tutti-frutti, alone and in combination in ET 24 hour and 48 hour at doses of 0.3 or 0.6 or 0.9 mL. The description of the results also presents the significant values of χ^2.

Table 2. Number and types of cellular abnormalities found in root meristem cells of *Allium cepa* treated with water and synthetic food flavorings cookie and tutti-frutti at doses of 0.3; 0.6 and 0.9 mL, at the exposure times of 24 and 48 hours.

Cookie Flavoring							
Dose (mL)	ET	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronuclei	Binucleate cell	TCA
	CO	01	00	00	00	00	01[a]
0.3	24h	25	27	13	51	00	116[b]
	48h	19	39	11	48	11	128[b]
	CO	00	00	00	01	00	01[a]
0.6	24h	22	15	29	43	18	127[b]
	48h	18	22	33	37	12	122[b]
	CO	00	00	00	00	01	01[a]
0.9	24h	13	47	19	11	00	90[b]
	48h	00	00	00	13	00	13[c]
Tutti-Frutti Flavoring							
Dose (mL)	ET	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronuclei	Binucleate cell	TCA
	CO	00	00	00	01	00	01[a]
0.3	24h	54	39	11	59	02	165[b]
	48h	43	37	19	74	00	173[b]
	CO	00	00	00	01	00	01[a]
0.6	24h	22	30	27	43	00	122[b]
	48h	23	31	21	48	00	123[c]
	CO	00	00	00	01	00	01[a]
0.9	24h	20	49	33	52	00	154[b]
	48h	19	42	38	62	00	161[b]
Cookie Flavoring + Tutti-Frutti Flavoring							
Combined doses (mL)	ET	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronuclei	Binucleate cell	TCA
	CO	00	00	00	01	00	01[a]
0.3+0.3	24h	29	59	27	78	16	209[b]
	48h	21	48	02	21	02	92[c]
	CO	00	00	00	01	00	01[a]
0.6+0.6	24h	25	17	13	47	00	102[b]
	48h	00	01	01	21	00	23[c]
	CO	00	00	00	01	00	01[a]
0.9+0.9	24h	23	29	20	31	00	103[b]
	48h	00	03	00	09	00	12[c]

ET –Exposure Time; CO – Control; TCA – Total Cell Abnormalities. Within the same treatment, TAC values followed by diffe rent letters are significantly different at 5% by χ^2 test.

The results in Table 2 indicate that the doses of cookie and tutti-frutti additives, for both forms of assessment, alone and in combination, resulted in a significant number of micronuclei and mitotic spindle changes, such as colchicine metaphase, anaphase and telophase bridges in meristematic cells of *A. cepa* roots. Therefore, in the present study, doses of cookie and tutti-frutti flavorings, as well as treatments combining such substances were genotoxic. Still in Table 2, for the three combination treatments at ET 48 hour, the number of cell changes was significantly lower compared to the number verified for their ET 24 hour. This result confirms the results described in Table 1, in which the number of dividing cells for the mentioned treatments was significantly lower in relation to their specific ET 24 hour.

Regulations of food surveillance agencies EFSA and ANVISA do not explain specifically what compounds and concentrations are flavoring additives. This information is also not available on the labels of flavoring solutions marketed or on websites specialized in the sale of these substances. Despite being limited the number of toxicity studies, at the cellular level, of food flavorings found in the scientific literature, there are some cytotoxicity evaluation studies of some chemical constituents of diluents and preservatives found in the composition of these microingredients. Such compounds are allowed and mentioned in technical documentation of food safety agencies.

Among them, stands out benzyl alcohol, diluent responsible for maintaining uniformity and facilitating incorporation and dispersion of flavor concentrated in food products. An analysis of the action of this diluent at the cellular level conducted by Demir, Kocaoglu and Kaya (2010) found that this alcohol, at high concentrations, led to significant damage to the mitotic spindle and therefore cell division in human peripheral blood cells. Other diluent commonly used in the formulation of flavorings is the diacetyl (2,3-butadione). Whittaker, Clarke, San, Begley and Dunkel (2008) reported, in gene mutation assay in rat lymphoma, that this compound caused significant damage to loci on chromosome 11 of these cells, causing loss of expression of genes for thymidine kinase in these animals. Additionally, More et al. (2012) verified that the diluent diacetyl had the potential to replace thymine with guanine in euchromatin regions and cause the disruption of hydrogen and disulfide bonds in the tertiary structure of enzymes involved in the cell division process.

Preservatives in food flavorings include potassium benzoate, sodium benzoate and potassium nitrate (Brasil, 1999), which, according to Mpountoukas, Pantazaki, Kostareli, Christodoulou, and KarelI (2010) and Zeguin et al. (2011), were clastogenic, mutagenic, and cytotoxic to normal human peripheral blood cells. Also present are boric acid, citric acid, potassium citrate and sodium citrate (Brasil, 1999), which led to a significant reduction in the cell division index of root meristem cells of *A. cepa*, proving to be cytotoxic to this test system (Türkoğlu, 2007).

For food flavorings, the only class of compounds with usage restrictions by regulatory bodies to some of its constituents is the extraction solvent class, where the agaric acid, aloin, beta-azarone, berberine, coumarin, hydrocyanic acid, hypericin, pulegone, quassine, safrole and isosafrole, santonin and tuyona alpha and beta have maximum tolerable limits discriminated in documents (Brasil, 1999; Brasil, 2007). In the meanwhile, for the manufacture of any food flavoring, it is necessary to join more than 20 classes of chemical compounds (Konishi et al., 2011; Xu et al., 2013).

Therefore, from the results obtained, along with the evaluation of toxicity at the cellular level already performed, while the use of aroma and flavor additives is permitted by EFSA and ANVISA, there is an urgent need for more detailed studies in the medium and long term, to determine properly the cytotoxicity of these substances and/or classes of chemical compounds that constitute them. It is worth mentioning that from cyto- and toxicological evaluation, in the short and medium term, developed in the 80s, food flavorings sparteine, allyl hexanoate and quinine have been banned for use in processed foods by ANVISA in the early 90s.

Conclusion

The cookies flavoring and combination treatments were cytotoxic and genotoxic, and the tutti-frutti flavoring, although non-cytotoxic demonstrated a genotoxic potential.

Our findings show the great need for more effective participation of food surveillance agencies as for the possible cytological and toxicological risks of flavoring additives to consumers, with emphasis on the flavorings cookie and tutti-frutti.

Similar studies can effectively assist health surveillance agencies to rethink and/or reorganize the content present in the normative documents of the agencies responsible for the regulation of these food additives.

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