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Cytotoxic, genotoxic and mutagenic potential of UHT whole milk

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Abstract

This study evaluated the action at the cellular level of long life whole milk, full type of six renowned companies operating in the Brazilian market, as well as in other South American countries. The evaluation was performed using root meristem cells of *Allium cepa* L., at exposure times 24 and 48 hours, directly in milk products marketed. The results indicated that all the milk samples reduced root meristem cell proliferation, proving, in this study, a significant cytotoxic effect. Still, exposure to milk resulted in a significant frequency of mitotic spindle changes in meristem cells, characterizing these foods as genotoxic and mutagenic under the study conditions. It can be concluded that the long life milk samples caused significant genetic instability to cells of the examined tissue. The results obtained for cytotoxic, mutagenic and genotoxic action of these long life milks are of great relevance because, to date, there are no published toxicity studies on such foods and food additives present in the composition.

Keywords: ultra-pasteurized milk; cell division; mitotic spindle alterations; meristematic tissue.

Practical Application: Verification of cell toxicity UHT milk in intense proliferation tissue.

1 Introduction

Bovine milk is considered a staple food because it contains, naturally and abundantly, biomolecules, such as water, glycides, proteins, unsaturated lipids, vitamins and minerals essential for the proper functioning of the body (Taffarel et al., 2013). However, despite nutritional importance, this food is highly perishable as it also is characterized as an excellent medium for the proliferation of undesirable microorganisms that, upon release of toxins, cause significant changes in taste, smell, color and, hence, nutritional properties (Aguiar et al., 2015).

In this way, to provide a healthy dairy product without contaminants, with extended shelf life and no preservative additives or micro ingredients, flavorings and colorings, food manufacturers have developed effective methods for quality control and preservation, such as the *ultra-high temperature* (UHT) technique, also known as ultra-pasteurization (Lima et al., 2009; Domareski et al., 2010). Raw milk subjected to this procedure is commercially known as UHT milk or long-life milk, which, based on the content of lipids is classified in whole, semi-skimmed or skimmed milk; and the first type is the most consumed by the population (Domareski et al., 2010; Souza et al., 2014). In Brazil, these dairy products are regulated by the Ministry of Agriculture, Livestock and Supply, through the Technical Regulation of Identity and Quality of long life milk - Ordinance Number 146 of 1995 (Brasil, 1995; Souza et al., 2014). This regulation was developed based on determinations of *Codex Alimentarius*, a body that regulates general rules for chemical composition, safety and food labeling worldwide (Brasil, 1995; Pflanzer et al., 2010).

However, although it is not attributed to long life milk, flavoring, color and preservative additives - micro ingredients scientifically proven to be toxic at the systemic and cellular levels (Marques et al., 2015; Moura et al., 2016; Sales & Peron, 2016) – it is added to these food wetting, stabilizer and antioxidant agents which, among other features, have the function to preserve the texture and homogeneity and ensure no oxidation of milk, especially after opened for consumption (Aguiar et al., 2015; Taffarel et al., 2013). Bodies, such as *Codex Alimentarius* and the National Health Surveillance Agency (ANVISA), point out in their technical regulations the constant need for toxicological studies on the acute effect of food microingredients in general, and especially of foods containing these compounds, since many food additives, such as those with wetting, stabilizing and antioxidant action, have not been evaluated for their cytotoxic, genotoxic, mutagenic and carcinogenic potential (Gomes et al., 2013; Oliveira et al., 2013; Marques et al., 2015; Moura et al., 2016; Nunes et al., 2016). Also, they emphasize that the results of toxicological analyses are the basis for preparation or modification of documents that regulate the basic composition and the daily intake rate or consumption of semi-processed and processed foods (Brasil, 2007; Moura et al., 2016; Sales & Peron, 2016). Nevertheless, in a broad search in the scientific literature, it was found no studies evaluating toxicity of ultra-pasteurized milk beverages.

Root meristems of *Allium cepa* L. (onion) are regarded in the scientific community as an effective bioassay for the assessment of acute toxicity at the cellular level of chemical compounds, since they have low chromosome number (2n = 16), which favors detection of chromosomal or clastogenic changes, alterations in the mitotic spindle or aneugenic activities, and changes in cell proliferation (Türkoğlu, 2007; Neves et al., 2014; Bianchi et al.,

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2015). This test system is internationally accepted by research agencies as an assessment tool with accurate sensitivity for analysis of cytotoxicity, genotoxicity and mutagenicity of the substance of interest, since the results obtained mostly show satisfactory similarity to those obtained via animal testing systems and cell cultures (Herrero et al., 2012; Lacerda et al., 2014; Tabrez et al., 2011; Gomes et al., 2013; Oliveira et al., 2013; Campos-Ventura et al., 2016; Moura et al., 2016; Santana et al., 2016; Bezerra et al., 2016; Sales & Peron, 2016; Nunes et al., 2016).

Given the addressed context, the present study aimed to evaluate, in root meristem cells of *A. cepa*, the cytotoxic, genotoxic and mutagenic potential of UHT whole milk of six relevant companies in the Brazilian food market, as well as in other countries in South America.

2 Material and methods

2.1 Treatment solutions

Samples of UHT milk of the six renowned companies operating in the Brazilian market, as well as in other South American contries, hereinafter referred to as A, B, C, D, E and F, respectively - were acquired in the retail market in the city of Picos, state of Piauí, Brazil. We were careful to check the shelf life period of the products and the integrity of packaging. Analyses of toxicity were carried out directly in the milk marketed in packaging.

2.2 Test of cytotoxicity, genotoxicity and mutagenicity with Allium cepa L.

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 milk sample, it was set up an experimental group with five onion bulbs. Before placing the roots in contact with their respective milk samples (treatments), some roots were collected and fixed to serve as control of the bulb itself. Then, the remaining roots were returned in their respective treatments for 24 hours, procedure called 24-hour exposure time.

After 24 hours, some roots were collected and fixed. Next, the remaining roots of each bulb returned to their treatments where they remained for additional 24 hours, which was called 48-hour exposure time. Subsequently, roots were again collected and fixed. 24- and 48-hour exposure times were chosen to evaluate the effect of long-life milk on more than one cell cycle. Roots were fixed in Carnoy 3: 1 (ethanol: acetic acid) for 24 hours. On average, three roots per bulb were taken in each collection.

Slides, on average, 03 per bulb, were prepared following the protocol proposed by Guerra & Souza (2002), and analyzed under a light microscope at 40× magnification. For each onion bulb, we examined 1,000 cells, totaling 5,000 cells for each control, 24- and 48-hour exposure times of each treatment group. In this way, for each milk sample, we analyzed 15,000 cells. These 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. MI values were used as a parameter for the determination of cytotoxicity

Mutagenicity and genotoxicity milk samples was also evaluated by frequency of micronuclei or clastogenic effects and colchicine metaphase, anaphase and telophase bridges, gene amplification, cells with adhesions, nuclear buds and multipolar anaphases, called aneugenic alterations or mitotic spindle changes. For statistical analysis, we used the chi-square test (χ^2) at a probability level < 0.05.

3 Results and discussion

The results in Table 1 demonstrate that all the samples of UHT milk caused a significant reduction in cell division of root meristems, at 24- and 48-hour exposure times, when compared to their respective controls. Similarly, when confronted with each other, cellular division indices obtained for the 24- and 48-hour exposure times of each sample, we verified a statistically significant inhibition of cell proliferation. Therefore, considering the results in Table 1, it can be inferred that the long life milk evaluated under the conditions of this study, have significant cytotoxicity to the test system used, which was more pronounced and significant with increasing exposure time

The cytotoxic potential of chemical compounds or substances can be determined by increasing or decreasing the mitotic index of tissues exposed to them (Fernandes et al., 2007). According to Caritá & Marin-Morales (2008), mitotic index lower than that of the negative control indicates the presence of agents whose toxic action affects the growth and development of exposed organisms. Complementing these authors, Gomes et al. (2013), Marques et al. (2015), Sales & Peron (2016) and Moura et al. (2016) claim that the inhibition of cell proliferation triggered by cytotoxic compounds in tissue with intense cell proliferation and with normal performance or without cellular changes, such as those used in this study for assessment of UHT milk toxicity, is quite harmful to the organism, since it possesses the property of inhibiting or limiting the replenishment of cells, altering the production of proteins and resulting in malfunction of the organ where it is located (Gomes et al., 2013; Marques et al., 2015; Sales & Peron, 2016; Moura et al., 2016).

The results shown in Table 2 indicate that all the samples of long life milk analyzed, for the 24-hour exposure time, have significantly induced cell changes in the meristematic tissue of roots. However, for all dairy products analyzed, the number of alterations verified for the longer exposure time was statistically lower than the results for the 24-hour exposure time. The reduction in cellular changes observed for longer exposure time corroborates the results of cell proliferation presented in Table 1, once all milk samples dramatically reduced cell division at the 48-hour exposure.

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Further, UHT milk products analyzed, at the 24-hour exposure time, have induced the formation of colchicine metaphase or C- metaphase in meristem cells, especially at the 24-hour exposure time (Table 2). The significant presence of mitotic spindle alterations shows that the evaluated milk were genotoxic to root cells. In agreement with Aissa et al. (2012), these disorders in tissues exposed to genotoxic agents evidence that such compounds primarily affect the integrity of the nuclear spindle, thus causing the improper alignment of chromosomes at the equatorial plate during mitosis and impeding the normal progress of the cell cycle.

At the shortest exposure time, we observed anaphase bridges, multipolar anaphases and telophase bridges (Table 2). According to Fernandes et al. (2007), bridges found in cells at anaphase and/or telophase occur by the action of chemical agents that significantly affect the mitotic spindle during

Table 1. Number of cells in each phase of cell cycle observed in root meristem of *Allium cepa* exposed for 24 and 48 hours to samples of whole long life milk of six food companies - referred to as A, B, C, D, E and F. In each treatment, significant values of χ^2 are presented.

		MITOTIC INDEX						
Company	ET	NIU	P	M	A	T	NDC	MI (%)
	CO	3943	513	232	150	162	1057	21.4[a]
A	24 h	4749	150	40	29	32	251	5.0[b]
	48 h	4955	42	03	00	00	47	0.9[b]
	CO	4363	198	200	186	53	637	12.7[a]
B	24 h	4859	49	29	32	31	141	2.8[b]
	48 h	4967	29	04	00	00	33	0.7[c]
	CO	3702	1004	135	105	54	1298	26.0[a]
C	24 h	4492	304	91	94	19	508	10.2[b]
	48 h	4819	152	29	00	00	181	3.6[c]
	CO	3901	841	109	78	71	1099	22.0[a]
D	24 h	4655	199	74	31	41	345	6.9[b]
	48 h	4976	24	00	00	00	24	0.5[c]
	CO	4282	289	243	108	78	718	14.4[a]
E	24 h	4850	44	39	54	13	150	3.0[b]
	48 h	4979	21	00	00	00	21	0.4[c]
	CO	3984	655	180	90	91	1014	20.3[a]
F	24 h	4816	81	41	53	09	184	3.7[b]
	48 h	4973	27	00	00	00	27	0.5[c]

ET – Exposure time; CO – Control; NIU – Number of cells in interphase and undifferentiated cells; P – Prophase; M – Metaphase; A – Anaphase; T – Telophase; MI – Mitotic Index; NDC – number of dividing cells. MI values followed by different letters in the same evaluated milk sample are significantly different by χ^2 test at 5%.

Table 2. Number of cellular alterations in root meristem of *Allium cepa* exposed for 24 and 48 hours to samples of whole long life milk of six food companies - referred to as A, B, C, D, E and F.

Company	ET	Colchicine metaphase	Anaphase/ Telophase bridge	Multipolar anaphase	Micronucleus	NCA
	CO	00	00	00	01	01[a]
A	24h	13	09	11	29	62[b]
	48h	00	00	00	01	01[a]
	CO	00	01	00	00	01[a]
B	24h	08	09	14	13	44[b]
	48h	00	00	02	00	02[a]
	CO	00	01	00	00	01[a]
C	24h	13	09	19	10	51[b]
	48h	00	00	00	03	03[a]
	CO	01	00	00	00	01[a]
D	24h	04	11	11	13	39[b]
	48h	00	00	00	01	01[a]
	CO	01	00	00	00	01[a]
E	24h	08	09	00	16	33[b]
	48h	00	00	02	00	02[a]
	CO	01	00	00	00	01[a]
F	24h	13	07	10	09	39[b]
	48h	00	00	00	01	01[a]

CO – Control; ET – Exposure time; NCA – number of cellular alterations. Values followed by different letters in the evaluated milk sample are significantly different by χ^2 test at 5%.

nuclear division, causing the drift of whole chromosomes at the end of cell division. In turn, Leme & Marin-Morales (2008) point out that the multipolar anaphases are also due to spindle malfunctioning, caused by genotoxic agents, which triggers an irregular distribution of chromosomes during separation of chromatids. Furthermore, ultra-pasteurized milk also induced, by virtue of the aforementioned mitotic spindle alterations, a significant frequency of micronuclei (Table 2). Fernandes et al. (2007) reported that such alterations, characterized by loss of chromatin, are formed during telophase when the nuclear envelope is reconstituted in the daughter cells.

According to Leme & Marin-Morales (2008), the significant presence of mitotic spindle alterations, as observed here by the action of long life milk, represents an important parameter of genotoxicity and mutagenicity of compounds or substances of interest. Thus, the data obtained with root meristem cells of *A. cepa* show that the dairy products evaluated have significant potential to cause toxicity at the cellular level. This result indicates that these foods must be soon assessed in bioassays, physiologically more complex, as in animals, since, according to Queiroz et al. (2013), cellular changes, when expressively present, as evidenced in this study, when observed at a significant frequency in animal tissue, have great potential to promote tumors, once there is a positive correlation between the increased frequency of micronuclei and development of cancer in mammals.

As previously mentioned, in the literature, we have not found studies evaluating the cytotoxic, genotoxic and mutagenic potential of long life milk, as well as the microingredients added to the composition. However, it is important to mention that there is great concern on the part of health professionals and food surveillance agencies as to the addition of chemical compounds not allowed by law by the relevant regulatory bodies (Mareze et al., 2015; Rocha et al., 2015). These substances are added in an attempt to maintain specific organoleptic properties, to increase the overall yield and therefore the profit made on the sale of these foodstuffs. Among the added compounds, stand out preservatives and alkylating agents (Abrantes et al., 2014). According to information available in the literature, the most commonly used preservatives are formaldehyde and/or hydrogen peroxide, as they help to eliminate much of the microbial flora of the milk, thus extending the shelf life of these foods. Meanwhile, the major chemical compounds used as alkylating agents include caustic soda and/or bicarbonate, for assist in maintaining the homogeneity and no oxidation of the milk.

Such chemicals used in milk adulteration have been widely studied for their toxic potential and showed significant toxicity at the cellular level. Nevertheless, it is very important to emphasize that brands and even food companies were not informed in studies that check fraud made in long life milk, which does not allow to suggest that the results obtained in this study occurred as a result of adulteration.

4 Conclusion

All the analyzed milk samples caused, significantly, reduction in cell division and mitotic spindle changes, proving to be cytotoxic, genotoxic and mutagenic.

The results obtained on the cytotoxic, mutagenic and genotoxic at the cellular level of long life milk are of great importance because, so far, there are no published studies on toxicity involving such foods.

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