

Cytotoxic and genotoxic potential of industrialized powdered milk for infants and young children

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ABSTRACT. This study aimed to evaluate the cytotoxicity and genotoxicity and determine the LC₅₀ concentration of powdered infant formulas widely marketed in South American countries. To this, milk samples, called as A, B, C and D, were analyzed in root meristem cells of *Allium cepa*, at concentrations of 0.075; 0.15 and 0.30 g mL⁻¹, for 24 and 48 hours; and through cell viability in culture of normal line cells, via MTT test, for 24 hours, in the concentrations 0.018; 0.0375; 0.075 and 0.15 g mL⁻¹. In *A. cepa*, all dairy products in the three concentrations caused significant inhibition of cell division in the meristems within the first 24 hours of exposure. In the *in vitro* evaluation, all milk formulas at 0.15 g mL⁻¹, as well as milk A at a concentration of 0.037 g mL⁻¹, C at 0.075 g mL⁻¹ and D at 0.037 g mL⁻¹, significantly reduced the cellular viability of the cell culture exposed to the foods studied, being potentially toxic. The milk A was considered the most toxic, with LC₅₀ of 0.031 g mL⁻¹, and B as the least toxic, with LC₅₀ of 0.15 g mL⁻¹. Therefore, the milk evaluated caused significant instability in cells of the test systems used and were characterized as cytotoxic.

Keywords: Infant formula; cell division; meristematic tissue; cell viability; LC₅₀.

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Introduction

Infant formulas of powdered or dehydrated milks are artificial foods used under specific conditions as partial or total substitutes for breast milk (Kiriyan et al., 2016). Such foods are composed of proteins, saturated lipids, linoleic acid, dietary fiber, mineral salts, vitamins and synthetic or artificial micro-ingredients like flavoring, acidity regulators and thickeners (Brasil, 1993; Brasil, 2001; Codex Alimentarius, 2014; Zou, Pande, & Akoh, 2016). In Brazil, infant milk is regulated and authorized for consumption and commercialization by the National Health Surveillance Agency (ANVISA), through the Brazilian Standard for Marketing of Foods for Infants and Young Children (Monteiro, 2006, Brasil, 2007). This regulation was based on determinations of Codex Alimentarius, which regulate the general rules of chemical composition, safety and labeling of processed foods worldwide (Codex Alimentarius, 2014; Prosser, McLaren, Frost, Agnew, & Lowry, 2008).

However, the food safety agencies European Food Safety Authority (EFSA), ANVISA, and the Codex Alimentarius declare that synthetic micro-ingredients, although essential for the manufacture of processed foods such as artificial infant milk, raise many questions with respect to its potential cytotoxic, genotoxic and mutagenic effects, since studies on the cellular toxicity of these compounds are little found in the scientific literature (Prosser et al., 2008; Brasil, 2007; Sales et al., 2016). In addition, professionals in different health areas claim that food additives contribute significantly to impoverishment of the diet and to the triggering or potentiation of pathologies, especially in children, such as disturbances in the functioning of the digestive tract, neurological problems, allergic reactions, diabetes, cardiovascular problems and cancer (Brasil, 2009; Konishi, Hayashi, & Fukushima, 2014).

Thus, there is relevance and urgency for research that, through suitable bioassays, evaluate the cytotoxic and genotoxic effects of foods added with synthetic micro ingredients. These evaluations are necessary for the promotion of food safety, since, according to Carvalho, Sales, and Peron (2017) and the development of the most common types of cancer results from the interaction between endogenous and environmental

factors, especially the diet when it is made up of over-industrialized foods. Nevertheless, after a thorough search in the scientific databases, no cellular toxicity assessment studies related to industrialized dehydrated milk, in general, were found.

Plant bioassays are considered satisfactory for monitoring the cytotoxic effects of chemical compounds (Carvalho et al., 2016) and meristematic root cells of *Allium cepa* (onion) have been indicated as an efficient test organism in the evaluation of cellular toxicity (Caritá & Marin-Morales, 2008), such as for example artificial food additives (Türkoğlu, 2007; Lacerda, Malaquias, & Peron, 2014) and processed foods (Türkoğlu, 2007; Carvalho et al., 2017). The *A. cepa* bioassay has excellent kinetic properties of proliferation and large and few chromosomes ($2n = 16$), which facilitates analysis for detection of changes in the cell cycle (Herrero et al., 2012; Neves, Ferreira, Lima, & Peron, 2014). It also allows the verification of alterations in the cell division index (mitotic index), as increase or reduction in the proliferation of root meristem cells exposed to chemical compounds of interest (Herrero et al., 2012). Additionally, it is important to note that the *A. cepa* system is validated worldwide by several researchers who, together with the evaluations made through this bioassay, perform tests on animals and cell culture, claiming that, in most cases, there is a significant similarity between the results obtained (Caritá & Marin-Morales, 2008).

Another important method for assessing cytotoxicity is the cell viability observed in culture of cells exposed to compounds or substances of interest (Marques et al., 2015; Carvalho et al., 2016). Such viability can be measured via reduction of the MTT (3-(4,5-dimethylthiazole-yl)-5-diphenyltetrazolium bromide), also called tetrazolium salt, through the enzymatic complex pyruvate dehydrogenase present in the mitochondria matrix. This complex transforms the tetrazolium salt into final metabolizing products called formazan crystals, which can be measured quantitatively. Thus, the MTT test relies specifically on mitochondrial functionality, allowing the determination of cell viability or metabolism in cell lines against chemical compounds (Marques et al., 2015; Bahuguna, Khan, Bajpai, & Kang, 2017). By means of this test, it is also possible to set the concentration of a given agent which results in the lethal concentration 50 (LC_{50}), which represents the value referring to 50% mortality of the population of exposed or treated cells. This value allows to estimate the acute cytotoxicity of a compound or substance (Marques et al., 2015).

Therefore, based on the context above, the aim was to evaluate, in root meristem cells of *A. cepa* and by means of the MTT test, the cytotoxicity and genotoxicity of artificial milks for infants (children up to six months of age) and for young children (over six months of age). The dairy products evaluated are marketed in countries of South America, including Brazil.

Material and methods

Powdered milk samples and concentrations for analysis

Powdered infant formulas of four different pharmaceutical industries, hereafter called as A, B, C, D, were purchased in a drugstore, representative of a national network, located in the city of Teresina, State of Piauí, Brazil, in October 2016. We were careful to check whether the milks were within the shelf life and the packaging were intact. The dairy products were packed in 400 g containers of the powdered product. A, B, and C were indicated for infants, and D, also known as follow-up milk, for infants in early childhood.

In the determination of the concentrations, the form of preparation and ingestion indicated on the labels of each milk product was used as a parameter. In all the packages, it was suggested to dilute 4.5 g powder in 30 mL water. Based on this information, the milk concentrations defined for study in *Allium cepa* and in cell culture were: 0.30, 0.15 and 0.075 g mL⁻¹, and 0.15; 0.075, 0.037 and 0.018 g mL⁻¹, respectively. To prepare the concentrations, we used distilled water. The experiments were conducted in triplicate for each concentration.

Cytotoxicity and genotoxicity test in root meristem cells of *Allium cepa*

Initially, the onion bulbs were placed in aerated flasks with distilled water at room temperature ($\pm 27^{\circ}\text{C}$) to obtain 2.0 cm long roots. For the analysis of each milk sample, we established an experimental group with five onion bulbs. Before placing the roots in contact with their respective samples (treatments), some roots were collected and fixed to serve as control of the bulb itself. Then, the remaining roots were placed in their respective treatments for 24 hours, a procedure called 24-hour exposure time.

After 24 hours, some roots were taken and fixed. After this procedure, the remaining roots of each bulb were returned to their respective treatments where they remained for another 24 hours, which is called 48-hour exposure time. Soon after, roots were again collected and fixed.

The exposure times 24 and 48 hours were chosen with the purpose of evaluating the effect of diluted powdered milk on more than one cell cycle. Roots were fixed in Carnoy 3: 1 (ethanol: acetic acid) for 24 hours. In each collection, three roots were taken per bulb.

On average, 03 slides per onion bulb were mounted 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 times 24 and 48 hours of each treatment group analyzed. In this way, for any milk sample, we analyzed a total of 15,000 cells. Cells were observed in interphase, prophase, metaphase, anaphase and telophase. From this analysis, we determined the mitotic index (MI), we used the following equation: (total number of cells in mitosis ÷ total number of cells analyzed) x 100. MI value was the parameter for determining the cytotoxic potential of milk samples under study.

The genotoxic potential of the concentrations of infant milk was evaluated by the frequency of micronucleated cells, C-metaphase, anaphase and telophase bridges, cells with adhesions, nuclear buds and multipolar anaphases. The statistical analysis was carried out by the Chi-square test (χ^2) at a probability level < 0.05, using the software BioEstat 3.0 (Ayres, Ayres Junior, Ayres, & Santos, 2007).

Cytotoxic activity evaluation by MTT method

The cytotoxicity of powdered milk samples against Vero cells was evaluated by the MTT (4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (Sigma-Aldrich, Germany) method, by counting the number of viable cells (Marques et al., 2015; Carvalho et al., 2016). The cells were cultured in 96-well plates (TPP, Trasadingen, Switzerland), at a density of 2×10^5 cells well⁻¹. After a 24 hours incubation period, at 37°C in an atmosphere of CO₂, the culture medium was removed and the cells were washed three times with serum-free L-15, and powdered milk diluted up to 200 µL mL⁻¹ in MM (L-15 medium with 2% serum) were added to the cells. Untreated controls were performed by the addition of 200 µL of MM.

The cells were then incubated for 24 hours. The medium was then removed and 50 µL of MTT solution (5 mg mL⁻¹) was added. Plates were reincubated for 4 hours. After that, the MTT solution was removed, 100 µL of DMSO was added to dissolve formazan crystals, and the plates were gently shaken, whereby crystals were completely dissolved. The solubilized product was quantified by spectrophotometry at 492 nm (reference at 620nm). Results were expressed as % viability cell considering absorbance control cells as 100% viable. Still, by means of the MTT test, we estimated the LC₅₀ (median lethal concentration) through the Probit Analysis method, at a probability < 0.05. In the graph, Significantly different from the control by Tukey's Test (p < 0.05).

Results and discussion

From the results in Table 1, it was observed that the cell division indices for the 24 and 48-hour exposure times of all analyzed milk samples, for all concentrations evaluated, when compared to the mitotic indices obtained for their respective controls, caused significant inhibition of cell division in the root meristems. Furthermore, for every concentration evaluated, the mitotic indices verified for the 24-hour exposure time were statistically similar to those observed for their specific 48-hour exposure time. Thus, the significant inhibition of cell division characterizes the dairy products, under the conditions of analysis established for this study, as cytotoxic for three concentrations evaluated (0.075; 0.15 and 0.30 g L⁻¹). It is noteworthy that for all milk samples, the concentration indicated on the labels as ideal for consumption (0.15 g mL⁻¹) was cytotoxic already in the first 24 hours of exposure. Importantly, the toxicity for all milk samples occurred immediately at the lowest concentration (0.075 g mL⁻¹) and at the shortest exposure time evaluated (24 hours). There were no significant changes in mitotic spindle and micronuclei in meristematic tissues exposed to infant formulas, demonstrating that the concentrations of milks analyzed were not genotoxic to root meristem cells of *A. cepa*.

Table 1. Number of cells in each phase of the cell cycle observed in *Allium cepa* root meristematic tissue exposed for 24 and 48 hours to powdered infant formula, termed as A, B, C and D. The milk samples or treatments were evaluated in the concentrations of 0.075; 0.15 and 0.30 g mL⁻¹.

Milk A									
TR	Conc	ET	TCII	P	M	A	T	TCD	MI (%)
A	0.075 g mL ⁻¹	CO	3528	410	352	367	343	1472	29.4 ^a
		24h	4915	26	21	30	85	85	1.7 ^b
		48h	4964	11	17	07	36	36	0.7 ^b
	0.15 g mL ⁻¹	CO	3378	502	401	418	301	1622	32.4 ^a
		24h	4945	22	31	01	01	55	1.1 ^b
		48h	4977	18	01	01	03	23	0.5 ^b
	0.30 g mL ⁻¹	CO	3044	631	571	503	251	1956	39.1 ^a
		24h	4981	07	09	03	00	19	0.4 ^b
		48h	4988	11	00	00	01	12	0.2 ^b
Milk B									
TR	Conc.	TE	TCII	P	M	A	T	TCD	MI (%)
B	0.075 g mL ⁻¹	CO	4102	204	292	178	224	898	17.9 ^a
		24h	4929	39	11	07	14	71	1.4 ^b
		48h	4975	13	09	03	00	25	0.5 ^b
	0.15 g mL ⁻¹	CO	3673	378	321	329	299	1327	26.5 ^a
		24h	4984	07	07	01	01	16	0.3 ^b
		48h	4986	11	02	01	00	14	0.3 ^b
	0.30 g mL ⁻¹	CO	3600	401	394	327	278	1400	28.0 ^a
		24h	4989	00	11	00	00	11	0.2 ^b
		48h	4986	01	13	00	00	14	0.3 ^b
Milk C									
TR	Conc.	TE	TCII	P	M	A	T	TCD	MI (%)
C	0.075 g mL ⁻¹	CO	3401	507	391	402	299	1599	32.0 ^a
		24h	4962	11	11	07	09	38	0.8 ^b
		48h	4986	04	00	00	10	14	0.3 ^b
	0.15 g mL ⁻¹	CO	3293	494	522	493	198	1707	34.1 ^a
		24h	4966	19	04	11	00	34	0.7 ^b
		48h	4989	07	01	01	01	11	0.2 ^b
	0.30 g mL ⁻¹	CO	3602	394	402	401	201	1398	27.9 ^a
		24h	4989	07	04	00	00	11	0.2 ^b
		48h	4987	09	00	00	04	13	0.3 ^b
Milk D									
TR	Conc.	TE	TCII	P	M	A	T	TCD	MI (%)
D	0.075 g mL ⁻¹	CO	3045	639	591	423	302	1955	39.1 ^a
		24h	4927	34	21	11	07	73	1.5 ^b
		48h	4970	09	13	01	07	30	0.6 ^b
	0.15 g mL ⁻¹	CO	3503	417	481	01	298	1497	29.9 ^a
		24h	4937	19	29	14	01	63	1.3 ^b
		48h	4981	04	13	01	01	19	0.4 ^b
	0.30 g mL ⁻¹	CO	3623	407	391	390	189	1377	27.5 ^b
		24h	4980	13	07	00	00	20	0.4 ^b
		48h	4984	03	09	01	04	16	0.3 ^b

TR – Treatment/Infant milk, TCII – Total number of cells in interphase and of undifferentiated cells; ET – Exposure Time; CO – Control; P – Prophase; M – Metaphase; A – Anaphase; T – Telophase; MI – Mitotic Index; TCD – Total number of dividing cells. MI values, within the same treatment, followed by different letters are significantly different at 5% by χ^2 test.

According to Santana et al. (2016), significant inhibition of cell division, as the one listed in Table 01, may be due to cell death due to disturbances such as the toxic action of chemical substances or compounds on the kinetics of cell division or damage to essential chromosomes. These events, according to Nunes, Sales, Silva, and Sousa (2017), cause a significant reduction in cellular replacement and alter the protein synthesis of the tissue or organ where they are. In addition, the antiproliferative effect observed can be explained, in agreement with Valavanidis, Vlachogianni, Fiotakis, and Loridas (2013) and Zilifdar et al. (2014), based on the fact that tissues with normal functioning, such as the root meristems of *A. cepa* used here, when exposed to compounds that alter vital cellular mechanisms, such as DNA replication and protein synthesis, have their cellular division significantly impaired.

In the tetrazolium reduction assay (Figure 1), powdered infant formulas A, B, C and D, at a concentration of 0.15 g mL⁻¹, as well as A, at concentrations of 0.075 and 0.037 g mL⁻¹; C and D, at 0.075 g mL⁻¹,

significantly reduced cell viability in the culture of cells exposed to the milks in question, proving their cytotoxic potential. These results corroborate those observed in *A. cepa* (Table 01), since it also interfered with the functioning and consequently with cell proliferation. It should also be observed that, as in *A. cepa*, in the *in vitro* test, all the dairy products analyzed were cytotoxic at a concentration of 0.15 g mL^{-1} , which is indicated commercially for consumption in the analyzed milk packages.

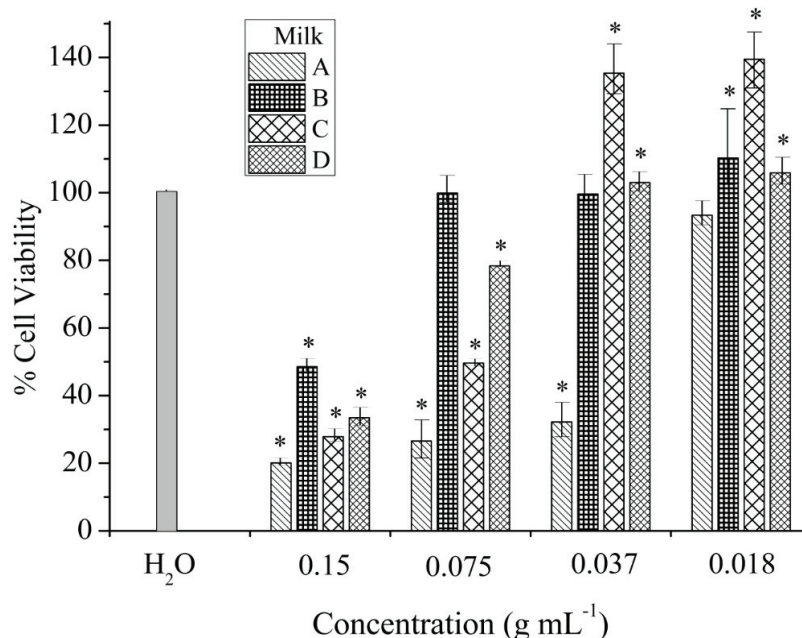


Figure 1. Cell viability in cell culture Vero exposed for 24 hours to samples of powdered infant formula diluted in distilled water and from four food companies, identified as A, B, C and D, at concentrations of 0.15, 0.075, 0.037 and 0.018 g mL^{-1} , evaluated by the MTT reduction test. *Significantly different from the control by Tukey's Test ($p < 0.05$).

Further, based on the results in Figure 1 and Table 2, it can be inferred that milk A was the most toxic among the analyzed products, with a LC_{50} of 0.031 g mL^{-1} , and milk B was the least toxic milk, with LC_{50} value of 0.150 g mL^{-1} . Results of LC_{50} concentration are widely used as parameters in the definition of doses concentrations⁻¹ of compounds or substances of interest to be evaluated in subsequent toxicity studies.

Table 2. LC_{50} values referring to toxicity of powdered infant formula, called as A, B, C, D, observed by MTT test.

Treatment	$\text{LC}_{50} (\text{g mL}^{-1})$
A	0.031
B	0.150
C	0.073
D	0.061

As previously mentioned, four classes of micro-ingredients are present in formulations of powdered milk for infants and children over six months of age (Brasil, 2009). These compounds include flavorings, additives responsible for providing and/or maintaining the aroma and flavor of industrialized foods (Oyelakin, Jaiteh, Salisu, & Adjivon, 2015). For infant milk formulas, only synthetic fruit and vanilla flavorings are permitted under Brazilian law (Brasil, 2001; Sales et al., 2016).

Although there are few studies on the toxicity at the cellular level of flavoring micro-ingredients in the scientific literature, studies have demonstrated cytotoxicity, genotoxicity and mutagenicity of some of the chemical constituents that are part of this additive, according to Codex Alimentarius (2014) for the formulation of synthetic flavorings additives in general. Among these constituents, stands out benzoic alcohol, responsible for facilitating the incorporation and dispersion of aromas in food products. Demir, Kocaoglu, and Kaya (2010) found that this alcohol promoted significant damage to the mitotic spindle, and, consequently, to cell division of human peripheral blood cells.

Another compound commonly found in flavorings is diacetyl (2,3-butanedione), whose function is to fix the aroma and flavor in industrialized foods (Brasil, 2009). Whittaker, Clarke, San, Begley, and Dunkel

(2008) evaluated the mutagenic potential of this chemical in a gene mutation assay using rats *lymphoma* cell line, L5178Y, and found that diacetyl caused significant damage to the loci on chromosome 11 and caused the functional loss of the thymidine kinase locus in these animals. In addition, More, Raza, and Vince (2012) found that high concentrations of diacetyl were mutagenic and had the potential to replace thymine with guanines. These changes had the property of breaking hydrogen and disulfide bonds of the tertiary structure of proteins, such as those involved in cell division, causing them to lose function. Moreover, for fruit and vanilla flavorings, Sales et al. (2016) evaluated the toxicity of these additives in root meristem cells of *A. cepa* and reported that these compounds were significantly cytotoxic and genotoxic to root meristems.

With respect to thickeners - additives which maintain or increase texture and consistency, as well as stabilize the proteins and vitamins of industrialized foods - authorized for use in milk for children, according to ANVISA (Brasil, 2009) standards, guar gum, locust bean gum, carob gum, garrofin gum, jatai gum, pectin and amino pectin. Toxicity assessment studies have shown that guar gum (Takahashi et al., 1994; Koujitani et al., 1997; Kappor, Ishihara, & Okubo, 2016), locust bean gum (Meunier et al., 2014) and pectin (Kang et al., 2006; Khotimchenko, 2010) were not cytotoxic to the test systems to which they were tested. For the other thickeners mentioned, no toxicity assessment research was found at the cellular level. Also based on the information provided by ANVISA (Brasil, 2009), acidity regulators - micro-ingredients that control acidity and alkalinity levels in foods - allowed for use in infant formulas include monosodium, trisodium, potassium and tripotassium citrates; potassium, calcium, sodium hydroxides, potassium bicarbonate, sodium carbonate and citric acid. No studies have been found evaluating cytotoxicity, genotoxicity and mutagenicity of thickeners in general for food.

Therefore, from the results obtained herein, although infant formulas, such as A, B, C and D, are authorized for consumption by different food safety agencies, and are an essential resource when breast milk is not sufficient or possible, there is a need for more detailed analysis, in the medium and long term, on different test systems and exposure times, on the toxic potential of these foods, as well as on the additives present in their compositions. Such evaluations, such as those carried out in the present research, are important for health promotion, once they represent important information for the elaboration or modification of the regulations that rule the basic composition and index of daily intake or consumption of semi-industrialized and industrialized foods in general.

Conclusion

The data obtained in the present study using the root meristem cells of *A. cepa* and the MTT test showed that the infant dairy foods evaluated had a significant potential to cause toxicity at the cellular level in the concentration 0.15 g mL^{-1} , which is indicated as ideal for consumption in the packaging of these products.

The observed results indicate the need to evaluate powdered infant formulas in physiologically more complex bioassays, as in animals, from treatments with longer exposure times, to check and deepen the results obtained here.

Our findings are of great relevance, since, to date, there are no published studies of cytotoxicity or LC_{50} concentration information involving such infant foods.

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