ASSESSMENT OF CYTOTOXICITY OF A CAFFEINATED SOFT DRINK USING *ALLIUM ASSAY*

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Abstract

One of the most consumed caffeinated soft drinks by millions of people globally is Coca-Cola. For healthy adults, caffeine consumption is relatively safe, but for some vulnerable populations, caffeine consumption could be harmful. Therefore, we considered this cytogenetic study to be appropriate for assessment of cytotoxicity effects of Coca-Cola in meristematic cells of plants, through Allium test. A. cepa has assayed to be one of the best model plants for standard use in cytological analysis of different toxins for their cytotoxicity and genotoxicity to plants and animals. The meristematic roots were treated with various concentrations of Coca-Cola (3, 5 and 10 ml/cl) for 8 hours, at room temperature, along to an untreated control. It was found that Coca-Cola induced a strong cytogenotoxic effect in meristematic cells of A. cepa as the concentration of Coca-Cola was increased. The clastogenic and aneugenic effect of the tested product was manifested by the decrease of mitotic index (12.5-3.5%) and the occurrence of several types of chromosomal and nuclear abnormalities (12.6-23.2%): bridges, laggards, stickiness and disrupted nucleus. These results suggest that the caffeinated soft drinks can be harmful to health and their regular intake must be avoided. The problem can arises when the consumption is regularized in everyday life, as, unfortunately, many people do.

Key words**: **Coca-Cola, Allium, cytotoxicity, clastogenic, aneugenic

INTRODUCTION

Soft drinks have been related to several harmful effects on health. In the last 30 years, the consumption of carbonated soft drinks has grown rapidly, especially among children and teenagers.

Coca-Cola (C-C) or Coke is one of the most consumed drinks in the world. In some countries, this drink is consumed instead of water. Although it is highly appreciated by consumers, there are studies showing that excessive consumption can lead to various health problems for them.

This soft drink with sugar and caffeine, which belongs of The Coca-Cola American Company, adds millions of sales every year.

The Coca-Cola Company is the world's largest soft drink company, the world's largest producer, distributor and marketer of soft drinks and syrups, and one of the largest US corporations. The company is best known for its most famous product Coca-Cola, invented by John Stith Pemberton in 1886 (https://www.ec.com).

The drink's name refers to two of its original ingredients: coca leaves and kola

nuts (a source of caffeine). The current formula of Coca-Cola remains a closely guarded trade secret; however, a variety of reported recipes and experimental recreations have been published. The secrecy around the formula has been used by Coca-Cola in its marketing as only a handful of anonymous employees know the formula (Kottasova, 2014).

Genotoxicological screening tests have been extensively used over time for assessing the health properties of compounds prior to being considered as safe substances. The potential of biotechnology products to elicit DNA damage in somatic or germ cells cannot be compared to that of the low-molecular-weight chemicals (De Souza and Bonciu, 2022a; De Souza and Bonciu, 2022b; Gocke et al., 1999).

Mexico is the country where the largest amount of sugary drinks is consumed, and the city of San Cristóbal De Las Casas ranks first in the consumption of Coca-Cola, but also in the highest rate of diabetes (https://stirileprotv.ro/stiri).

The consumption of caffeinated drinks is often intended entirely or partly for the physical and mental effects of caffeine. Synthetic caffeine is also added to soda and energy drinks (Ahluwalia and Herrick, 2015), which are commonly consumed by children and adolescents worldwide, and to other food and non-food products with niche markets for subsets of consumers, such as juice, chewing gum, water, cookies, hot sauce, candy, beef jerky, mints, syrup, waffles, shampoo, soap, lip balm, eye cream, body scrub, and body lotion. These products are primarily marketed with claims that they provide energy, alertness, or are “age-defying.” Last year, the FDA announced that it will begin investigating the safety of caffeine added to food products, with a special emphasis on children and adolescents (Nehlig, 1999).

The question that arises is whether caffeinated drinks affect the well-being of the body of the one who consumes it. Caffeine overdoses has been linked to serious health problems including arrhythmia, arterial fibrillation, myocardial infection which can lead to death (George and George, 2017). According to Ito et al., caffeine in soft drinks causes changes in the DNA repair system, which leads to disease in various organs, especially cardiovascular abnormalities and pancreatic cancer.

On the other hand, it is well known that there are several extra compounds in C-C, such as carbohydrate syrups, phosphoric acid (E-338) and class IV caramel colorants, but none of them has been reported as antioxidant (Luna et Aguilera, 2014, Mateo-Fernandez et al., 2016). Caffeine has been known to inhibit the activity of several related enzymes including ataxia telangiectasia mutated (ATM) kinase, rad-3-related (ATR) kinase and mammalian target to rapamycin kinases (Sarkaria et al., 1999).

As farming and agricultural studies have progressed, so have the number of studied stressors (Cotuna et al., 2021; Cotuna et al., 2022a, 2022b; Paraschivu et al., 2020). Food is key to health but sometime, some undesirable situations can disrupt the food system and humans relationship with food (Paraschivu et Cotuna, 2021). For example, the presence of antibiotics is prohibit in milk intended for human consumption to protect hypersensitive individuals from exposure to specific antibiotics (Colă and Colă, 2017, 2018, 2021). Sustainable food and agriculture assure all dimensions of sustainability: environmental, social and

economic (Cotuna et al., 2015; Partal and Paraschivu, 2020).

We considered this cytogenetic study to be appropriate for assessment of cytotoxicity effects of C-C by Allium test. *Allium* assay is one of the best models for standard use in cytological analysis but also environmental monitoring (Bonciu et al., 2018; Liman et al., 2022; Olaru et al., 2020; Roșculete et al., 2019). Also, the *Allium* test is well known and commonly used in many laboratories. Because the onion are easy to store and to handle and the meristematic root cells constitute a convenient system for macroscopic as well as for microscopic parameters (Bonciu, 2019; Bonciu et al., 2020; Liman et al., 2021).

MATERIALS AND METHODS

The biological material was represented by 10 medium sized onion bulbs, which were immersed in glasses with tap water for 72 hours, time required for the meristematic roots occurrence. When the meristematic roots reached the length of 1.5 cm, they were immersed in glasses with various concentrations of C-C (3, 5 and 10 ml/cl) for 8 hours, at room temperature. These concentrations were established based on the assumption that the tested soft drink can be consumed from moderate to excessive amounts. A number of nine onion bulbs were used for each treatment variant as well as an untreated control (one bulb) that was remain immersed in tap water.

The roots were processed according to the Feulgen protocol and staining with Schiff reagent. The microscopic slides were performed according to the squash method. 500 cells were counted for each variant.

Data were analyzed using SPSS, ver. 23.0. The analysis of variance (ANOVA) was used to assess the significant differences between control and each treatment. If there was a significant differences ($p<0.05$), the experimental data analyzed using Duncan's multiple range test.

The mitotic index was calculated using the following formula:

$$MI\ (\%) = \frac{\text{Total number of cells in division}}{\text{Total number of analysed cells}} \times 100$$

The index of the total abnormalities (TA) was also calculated:

$$TA\ (\%) = \frac{\text{Total number of aberrant cells}}{\text{Total number of cells in division}} \times 100$$

RESULTS AND DISCUSSIONS

Generally, the *Allium* test is a very useful tool for evaluating and ranking many chemicals with reference to their toxicity. The *A. cepa* test also enables the evaluation of different endpoints. Among the endpoints, chromosome aberrations have been the most used one to detect genotoxicity.

The mitotic index, chromosomal and nuclear abnormalities are used to evaluate citotoxicity to quantification mutagenicity of different chemicals.

From the point of view of the cytotoxicity effect induced by C-C to *A. cepa*, our results indicate that the MI decreased in all variants with increased C-C concentration (Table 1).

Table 1. Results from cytogenotoxicity testing of C-C in *A. cepa* meristematic roots

Time (h)	Conc. (ml/cl)	MI (% ± SD*)	S	B	L	DN	TA (% ± SD)
8	0(Ct)	12.5±0.9a	8	1	1	3	2.6±0.5a
8	3 (V1)	8.9±0.4a	23	5	9	12	9.8±1.9b
8	5(V2)	5.1±0.4b	37	7	12	25	16.2±0.8b
8	10(V3)	3.5±0.5c	58	10	16	32	23.2±1.2c

*Different letters in the same columns for each treatment concentration are statistically significant (P≤ 0.05)

MI=Mitotic index, SD=Standard deviation, S=Stickiness, B=Bridges, L=Laggards, DN=Disrupted nucleus, TA=Total abnormalities

Thus, the MI recorded values amounted to 12.5% (Ct), 8.9% (V1), 5.1 (V2) and 3.5 (V3) respectively. On the other hand, the

prophase, metaphase, anaphase and telophase of the mitotic division recorded lower values compared to the control variant, as the C-C concentration increased.

The tested soft drink induced a high number of mitotic aberrations and nuclear anomalies in the cells of *A. cepa*: bridges, laggards, stickiness and disrupted nucleus (Figure 1). The increase of TA was dependent on increasing C-C concentrations.

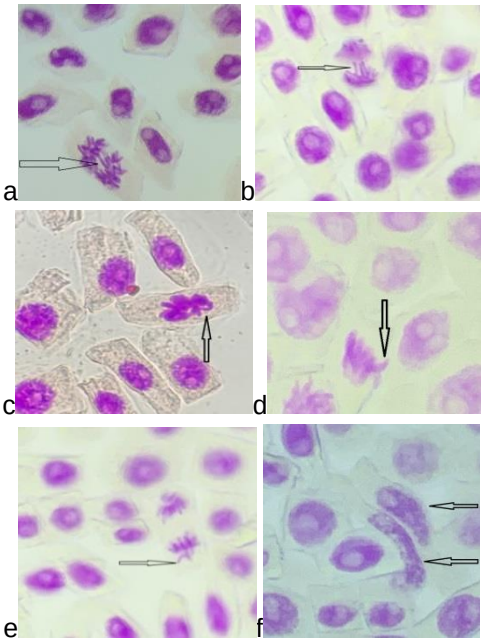

Figure 1. Different cytogenetic anomalies induced by C-C in *A. cepa* meristematic roots: bridge in anaphase (a, b); sticky metaphase (c) laggard chromosome (d, e); disrupted nuclei (f)

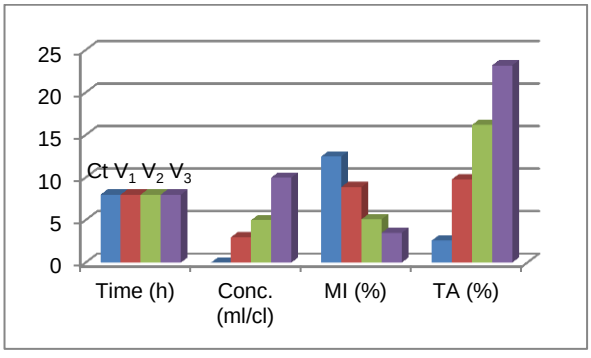

Figure 2. The decrease of mitotic index and the increase of cell aberrations in meristematic cells of *A. cepa* exposed to C-C

A correlation was observed between the mitodepressive effect and the percentage of chromosomal and nuclear aberrations induced by C-C in meristematic cells of *A. cepa* (Figure 2).

These results suggest that the caffeinated soft drinks can be harmful to health and their regular intake must be avoided.

The stronger cytogenotoxic potential was registered by C-C 10% (V3), where the TA percentage was of 23.2%, significantly higher than the control variant (2.6%). In case of this variant (V3), were identified 58 cells with sticky chromosomes, 10 bridge-type aberrations, 16 laggards and 32 cells with damaged nucleus.

A high cytogenotoxic effect was also observed in the case of the V2 variant, where the TA value was 16.2% and a number of 37 cells with sticky chromosomes, 7 bridge-type aberrations, 12 laggards and 25 cells with damaged nucleus.

At 3 ml/cl C-C concentration (V1), the TA percentage was of 9.8% and was identified 23 cells with sticky chromosomes, 5 bridge-type aberrations, 9 laggards and 12 cells with damaged nucleus.

MI is considered to reliably identify the presence of cytotoxic pollutants in the environment but also for cytogenotoxic potential of different chemicals (Liman et al. 2021).

As shown in mainstream literature, the decrease of the mitotic index value below 50% compared to the control variant leads to a sublethal effect, while below 22% it can cause lethal effects on test organisms. In our results, the high mitodepressive effect of the C-C described their cytotoxic potential in *A. cepa* meristematic roots.

In this study, the frequency of cells with sticky chromosomes increased with the

increase of C-C concentration. Actually, stickiness was the most frequent chromosomal aberration observed in root tips of *A. cepa* exposed to C-C. Other researchers suggest that sticky chromosomes reflect highly toxic effects and probably lead to cell death (Donghua et al., 1996).

Another aberration induced by C-C in the meristematic cells of *A. cepa* was bridges. Some authors conclude that chromosome bridges can produce tension that can cause the kinetochores at the end of the chromosome bridge to move away from the newly formed daughter nuclei, potentially resulting in the formation of micronuclei or abnormally shaped nuclei in the daughter cells (Pampalona et al., 2010; Stewenius et al., 2005).

Over the past decade, the introduction of new caffeine-containing food products, as well as changes in consumption patterns of the more traditional sources of caffeine, has increased scrutiny by health authorities and regulatory bodies about the overall consumption of caffeine and its potential cumulative effects on behaviour and physiology (Temple, 2017).

Moderate caffeine consumption among adults is considered to be around 300 mg per day. The caffeine content of Coca-Cola is approximately 24 mg per 250 ml serving, significantly less than the caffeine contained in the same amount of roasted coffee and also less than the caffeine content of 25-30 ml of espresso coffee.

However, the effects of caffeine on cellular growth in malignant cells are controversial. Thus, Ito et al. (2003) investigated the effects of caffeine on cell proliferation, cell cycle progression and induction of apoptosis in NB4promyelocytic leukemic cells containing wild-type p53. Caffeine suppressed the cellular growth of NB4 cells in a dose and time-dependent manner.

Temple et al. (2017) report that, for healthy adults, caffeine consumption is relatively safe, but that for some vulnerable populations, caffeine consumption could be harmful, including impairments in cardiovascular function, sleep and substance use.

According to Mateo, an apparent scarce data on the lack of C-C dose-dependent effect were observed at almost all parameters analysed at the individual, cell, and molecular levels. The authors only found a clear-cut dose-dependent effect when C-C is tested in the antitoxicity, cytotoxicity, and methylation bioassays (Mateo-Fernández et al., 2016). In this context, a threshold level of concentration may be needed to obtain some biological effects.

Nevertheless, studies assessing systematically the toxicological effects of cola beverages are scarce (Tamura et al., 1977, 1979) or showed contradictory results. Further substantial contributions in this sense are expected (Beckingham et al., 2005; Bier, 2005).

CONCLUSIONS

The Coca-Cola drink induced a high number of mitotic aberrations and nuclear anomalies in cells of *A. cepa*: bridges, laggards, stickiness and disrupted nucleus. The increase of total abnormalities was dependent on increasing Coca-Cola concentrations.

A correlation was observed between the mitodepressive effect and the percentage of chromosomal and nuclear aberrations induced by Coca-Cola in the meristematic cells of *A. cepa*.

The cytogenetic results suggest that the caffeinated soft drinks can be harmful to

health and their regular intake must be avoided. On the other hand, the occasional consumption of Coca-Cola probably does not involve high risks, but the problem can arises when the consumption is regularized in everyday life.

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