Genotoxic and Cytogenic Effect of Four Loko in Human Cells

Laura Ivonne Herrera Reyes. Ameyalli Flores Herrera, Emilio André Vázquez Hernández, high school students from Centro Universitario México, ivonne.herrera@cum.maristas.edu.mx, 94190328@cum.maristas.edu.mx y, 94190314@cum.maristas.edu.mx

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Supervisor: Biol. Laura Ivonne Herrera Reyes
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http://www.ayimi.org , info@ayimi.org

ABSTRACT

Four Loko® is a beverage which contains between 12% and 14% of alcohol, sugar, caffeine, taurine, wormwood and furfural, causing an intoxicated state accompanied with headaches, nausea, diarrhea, abdominal pain, general discomfort. For this reasons, one of the studies that must be realized is the toxicological test therefore in this project we evaluated the genotoxic effect of Four Loko® in lymphatic cells. Micronucleus are chromosomes fragments which are left out of the nucleus and can be caused by clastogen agents such as radiation that breaks chromosomes and aneuplodogen agents which damage mitotic spindle such as vincristine.

Key words: *Micronucleus, Four Loko®, giemsa, lymphocytes, clastogen, aneuplodogen*

1 Introduction

1-1 Justification

Four Loko® is an energetic beverageconsumed by young people in parties and social events, it contains 12% alcohol, wormwood, furfural and taurine between others. This beverage has been banned in several countries for its secondary effects. The deputy María Mercado Sánchez requested Cofepris to realize toxicologic studies for this beverage since many young people have been hospitalized by drinking it, and even launched an inciative to withdraw it of the Mexican market. Students from the CUM made a research to determine the lethal dosis of Four Loko® in Drosophila melanogaster, yet the higher concentration which could be tested was of 40% because the alcohol levels of the beverage exceed the tolerance levels of the fly. The SMART test revealed around 11 mutations with mwh and flare3 markers (Ferral, et al, 2019, P: 3-5). For this reason we decided to continue with the Project, evaluating its genotoxicity in lymphatic human cells using the micronucleus (MN) technique employed by Castillo and Fujita in 2011, which allows to determine the damage in cellular DNA (Fig.1).

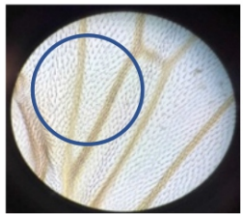

Fig. 1: Mutations in flies' trichrome

1-2 Problem Statement

Many alcoholic beverages that are sold in convenience stores like Four Loko® don't have preliminary toxicologic studies which confirm a secure intake, furthermore, they are sold unrestrictedly to minors which turns them into a health risk. This is the reason which lead us to raise the next project where we evaluate the genotoxic effect of Four Loko® in lymphatic cells by the determination of micronucleus (Fig. 2).

Fig 2: Four Loko Black

1-3 Hypothesis

If Four Loko® contains cutogenic and/or genetic substances, then we will observe in the lymphatic cell the presence of MN, if they are small then it will have clastogenic compounds and if they are big then it will have aneuploidogenic substances.

1-4 Research Purpose

- Evaluate the genotoxic and cytogenic effect of Four Loko® in lymphatic cells.
- Observe the behavior of the lymphatic cell which were exposed to the different Four Loko® concentrations.

2 Theoretical Framework

Four Loko is mainly consumed by Young people between 15 and 24 years old because of it easy access and unrestricted sale in 24 hours convenience stores. This sweet beverage has caused important reactions in the young people who drink it and some have even been emergency admitted to hospitals. In the section of "Nación" in the Mexican newspaper "El Universal" (07/17/2019) it says: Cofepris analyzed the content of this beverage and as a result of their analysis in batches L19338354 and L1751338 it was detected the presence of furfural in quantities outside de Official Mexican Norm NOM-142-SSA1/SCFI-2014 Alcoholic Beverages.

Furfural is an ethanol metabolite which in high quantities can cause: headache, nausea, diarrhea, abdominal pain and malaise in general, the preliminary results in reproductive studies and of feeding in mice and rats demonstrate the possibilities of birth and reproductive defects (Rivera, S. M. y Aguilera, R. J., 2000).

Micronucleus are spherical cytoplasmic corpuscles which are detected in the interphase, smaller and with the same characteristics of the cell nucleus; they are origintaed by the loss of chromosomic fragments or entire chromosomes

suring the cellular division and they have value in the diagnosis of genotoxicity (Castillo, Guevara-Fujita y Fujita, 2011, P: 1) (Fig. 4).

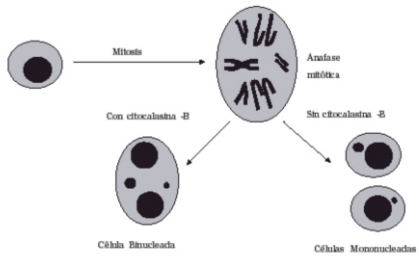

Fig. 3: Micronucleus formation by the loss of an entire chromosome and chromosomic acrocentric fragments in mitotic anaphase. The scheme shows the blockade with cytochalasin-B and the resulting formation of binuclear cells

Micronucleus may be caused by clastogenic agents (such as radiation that breaks chromosomes) and by aneuploidogenic agents that damage the mitotic spindle such as vincristine. Micronuclei are known in the field of hematology as Howell-Jolly bodies and their shape is generally round or almond-shaped, with a diameter of 0.4 to 1.6 microns. If the compound studied is a clastogen then a small micronuclei will be formed; however, if a large micronucleus is formed it will be an aneuploidogenic. (Zúñiga, G. G. & Gómez, M. B, 2006, P: 1- 3). The year 1999 was crucial for the MN test since the technique was validated worldwide and considered as an effective biomarker of DNA damage. In order to validate the technique an international human micronucleus program (HUMN: Human Micro Nucleus Project) was created to collect the basal frequencies of MN obtained in different laboratories around the world. (Zalacain, M., Sierrasesúmaga, L. y Patiño , A., 2005, P: 3,4).

3 Methodological Process of the Project Development

The research took place from October 7, 2019 to March, 3 2020 at the CUM's laboratory. The independent variable was the concentration of a commercial beverage known in the market as Four Loko® and the dependent variable was the presence of micronucleus in lymphocytes.5 ml of peripheral blood were extracted from 3 healthy donors and between the ages of 15 and 18 years old(Fig . 4).

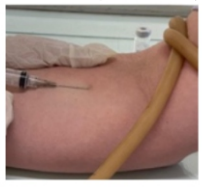

Fig. 4: Blood Extraction

0.5 ml of heparin were added to the blood sample and this was collocated in centrifuge tubes (Fig. 5).

Fig. 5: Tubes with Heparin

The samples were centrifuged for 30 minutes at 3000 rpm to separate the components by density (Fig. 6).

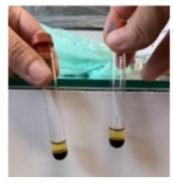

Fig. 6: Blood separation by density

The lymphocyte interface was separated and 5 ml of PBMAX (gibco) were added; then it was incubated at 37°C for 48 hours (Fig. 7).

Fig. 7: Addition of the culture medium

After 48 hours, 6 samples of Four Loko® black were formed and the concentration was added. In the first sample, 0.5 ml at 100%, in the second 0.5 ml at 80%, in the third 0.5 ml at 60%, in the fourth 0.5 ml at 40%, in the fifth 0.5 ml at 20%, and finally, the sixth where as positive control, mitomycin C (MMC-Signma MO503) was used and, as negative control, ethyl alcohol at 12% was added(Fig. 8).

Fig. 8: Four Loko concentrations are added

After 24 hours, 2 drops of colchicine were attached and it was incubated at 37°C for another 24 hours. 96 hours later, all the tubes were centrifuged at 3000 rpm for 5 minutes. The overlying was decanted and one drop from the beaker was collocated on a clean glass slide doing a smear (two repetitions in each sample).

Fig. 9: Tubes ready for the final decantation. The lymphocytes buttons are observed

The samples were set for 5 minutes with an acetic acid solution and methanol 1:1. Then, they were dyed with giemsa colorant at 5% for 8 minutes and they were looked through with a microscope at 100X using immersion oil.

Fig. 10: Staining of samples

IDN of binucleate cells was calculated, for genotoxicity the number of cells were divided with a micronucleus for concentration between the total cells quantified. For cytotoxicity the number of nuclei per multinucleated cells between the number of total cells.

4 Results

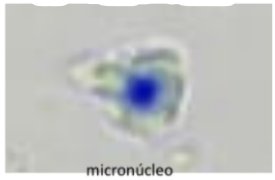

Fig. 11: Mononucleated cell, alcohol with water

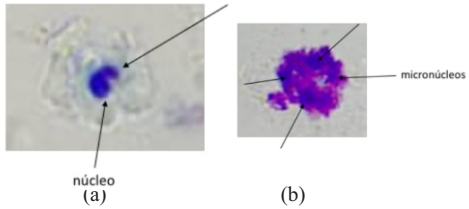

Fig. 12: a)Binucleated cell, b) Multinucleated cell, 100% concentration

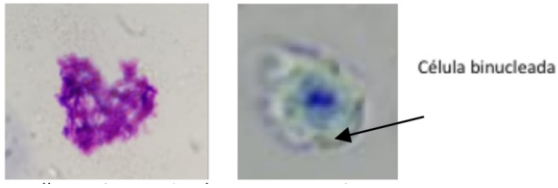

Fig. 13: On the left, cell with cromatine and on the right, binucleated cell with a 50% concentration

Table 1: Four Loko effect in human lymphocytes

Concentración	No. de células totales	Promedio por muestra	Células con núcleo alterado	Genotoxicidad	Citotoxicidad	IDN
Mitomicina C						
(+)	780	195	550	0.70512821		1.617
100%	436	109	280	0.64220183	0.800	0.823
80%	400	100	200	0.50000000	0.589	0.588
60%	450	113	150	0.33333333	0.432	0.441
40%	420	105	125	0.29761905	0.341	0.367
20%	370	92	98	0.26486486	0.250	0.288
(-)	400	100	34	0.085	0.090	0.100

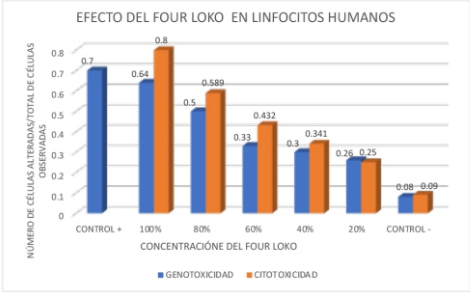

Fig. 14: Genotoxic and cytogenic effect of Four Loko

5 Analysis and Discussion

Photographs of the experiment allow to appreciate different micronucleus; the smaller correspond to clastogens substances and the bigger to aneuploidogens. (Fig. 14) shows that the genotoxic and cytogenetic of Four Loko® effect is observed in lymphocytes. We confirm our hypothesis since the presence of micronucleus in lymphocytes reveals the damage that Four Loko® causes to cellular DNA In Table (1), IDN concentration in this beverage is very high; it is almost 50% of mitomicyn. This is a substance highly mutagenic. We would like to make a spectrophotometric of gases to verify the real content of the substances included in this beverage and analyzed them separately.

5-1 Future Research Lines

This project opens an important line of investigation concerning people's health care. It is important that any food or product (medical or cosmetic) complies with the quality standards established by the norm to guarantee the safety of the consumers. Young population is considered a vulnerable group due to the tendency they have to follow "trending stuff" and "fashions" that could represent an irreparable damage to their reproductive and medical health since, nowadays, genotoxicty is being linked to some severe diseases such as cancer.

6 Conclusions

In all tested concentrations it is shown that Four Loko® is a toxic substance that alters cell division causing the rupture of mitotic spindle or the fragmentation of chromosomes generating multinucleated cells. Lymphocytes exposed to the different concentrations of Four Loko® present MN in all of the concentrations, therefore it contains aneuploidogenic and clastogenic substances which cause DNA cellular alterations. Four Loko® is a genotoxic substance, the presence of micronucleus demonstrates it and the concentration of the beverage is related to them since the higher the concentration, the bigger the number of multinucleated cells. Therefore, we accept our hypothesis, and we propose to make similar studies for every Four Loko® component.

We recommend to take it out of the market until further research is conducted, and to put all of its ingredients on its nutritional label for the public.

7 Acronym List

Cofepris – Federal Comission for Protection Against Sanitary Risk
MN – Micronucleus
mwh – multiple wing hairs. Flies line with cellular marker of trichomes in wings
flr3 – phenotype resistent
ADN – Deoxyribonucleic Acid
HUMN – Human Micro Nucleus Proyect
CUM – Centro Universitario México
SMART – Somatic Recombination and Mutation Test
NOM – Mexican Official Norm
IDN – Nuclear Division Rate

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