UNIVERSIDADE FEDERAL DO PAMPA CAMPUS URUGUAIANA
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THAIS PASQUALLI

IMUNOTOXICIDADE DE EDULCORANTES ALIMENTARES: RISCOS *VERSUS* BENEFICIOS.

Uruguaiana 2020

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Michel Mansur Machado

Coorientador: Prof. Dr. Luís Flávio Souza de Oliveira

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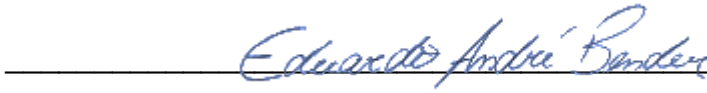
Banca examinadora:

Prof. Dr. Michel Mansur Machado (*Orientador*)
UNIPAMPA

Profa. Dra. Cristiane Casagrande Denardin
UNIPAMPA



Prof. Dr. Eduardo André Bender
UNIPAMPA

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“*Agradeça cada <u>não</u> que te fez olhar perspectivas e te fez buscar novos tantos caminhos*”.

Autor desconhecido

RESUMO

A busca de uma vida mais saudável fez com que o uso de adoçantes substitua o açúcar. De modo geral, os adoçantes diminuem a incidência de cáries, obesidade e doenças associadas, como diabetes e hiperlipidemia. No entanto, existem diversos questionamentos sobre a segurança desses adoçantes. Esse trabalho tem como objetivo analisar os efeitos citotóxicos, genotóxicos e mutagênicos dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni em concentrações semelhantes as utilizadas no consumo humano utilizando cultura de linfócitos humanos. Para tanto, a cultura celular foi preparada utilizando sangue de indivíduo auto-titulado saudável, coletado por punção venosa e as células de interesse foram separadas com Histopaque® 1.077. Foi realizada uma curva-efeito para estabelecer as concentrações a serem utilizadas, onde foi analisada a proliferação e viabilidade celular. Os parâmetros genotoxicológicos foram analisados pelo teste cometa e instabilidade cromossômica. Foram analisados também os efeitos dos adoçantes em subpopulações linfocitárias. De forma complementar, os adoçantes foram submetidos a testes computacionais. Através da curva dose-efeito as concentrações definidas foi de 1 µg/mL, 10 µg/mL e 50 µg/mL para cada adoçante. Nas análises de citotoxicidade notou que a Sucralose e Steviol causaram uma diminuição no número de linfócitos. Já com a Frutose e Sacarina, esta proliferação não foi afetada. Na viabilidade celular nenhum adoçante causou redução da quantidade de linfócitos, porém no teste com as subpopulações linfocitárias a Sacarina apresentou inibição de linfócitos $TCD3^+$, $TCD8^+$ e T dupla população; o Steviol mostrou inibição de linfócitos $TCD4^+$ e dupla população e os adoçantes Sucralose e Frutose apresentaram inibição de linfócitos $TCD4^+$, $TCD8^+$ e T dupla população. No teste cometa todos os adoçantes avaliados causaram danos ao DNA em pelo menos uma concentração testada. Já no teste de instabilidade cromossômica, somente a Frutose não gerou alterações. Pelo teste *In Silico,* foi possível propor que o principal mecanismo envolvido para o dano e/ou alterações no DNA está relacionado com a modulação da expressão gênica, interferindo em proteínas relacionadas à replicação, manutenção e reparo de dano ao DNA.

Palavras-Chave: adoçantes, genotoxicidade, mutagenicidade, citotoxicidade.

ABSTRACT

The search for a healthier life has made using sweeteners to replace sugar. In general, sweeteners decrease the incidence of cavities, obesity, and associated diseases, such as diabetes and hyperlipidemia. However, there are several questions about the safety of these sweeteners. This work aims to analyze the cytotoxic, genotoxic, and mutagenic effects of sweeteners saccharin, Sucralose, fructose and Stevia rebaudiana Bertoni in concentrations like those used in human consumption using human lymphocyte culture. For that, the cell culture was prepared using blood from a healthy self-titled individual, collected by venipuncture and the cells of interest were separated with Histopaque® 1,077. An effect curve was performed to establish the concentrations to be used, where cell proliferation and cell viability were analyzed. Genotoxicological parameters were analyzed by comet test and chromosomal instability. The effects of sweeteners on lymphocyte subpopulations were also analyzed. Besides, the sweeteners were subjected to computational tests. Through the dose-effect curve, the defined concentrations were 1 µg / mL, 10 µg / mL and 50 µg / mL for each sweetener. In the cytotoxicity analyzes, he noticed that Sucralose and Steviol caused a decrease in the number of lymphocytes. Already with fructose and saccharin, this proliferation was not affected. In cell viability, no sweetener caused a reduction in the number of lymphocytes, however in the test with lymphocyte subpopulations, saccharin showed inhibition of TCD3 +, TCD8 + and T double population lymphocytes; Steviol showed inhibition of TCD4 + lymphocytes and double population and sweeteners Sucralose and fructose showed inhibition of TCD4 +, TCD8 + and T double population lymphocytes. In the comet test, all sweeteners evaluated caused DNA damage in at least one concentration tested. In the chromosomal instability test, only fructose did not generate changes. By the *In Silico* test, it was possible to propose that the main mechanism involved for DNA damage and changes is related to the modulation of gene expression, interfering with proteins related to DNA damage replication, maintenance, and repair.

Keywords: sweeteners, genotoxicity, mutagenicity, cytotoxicity.

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LISTA DE ABREVIATURAS

n. – número

col. – colaborador

mg – miligramas

Kg – quilograma

g – força gravitacional

min – minutos

ºC – graus Celsius

CO_2 – Dióxido de Carbono

mL – mililitro

µg – micrograma

CL50 – concentração letal de 50%

mm^3 – milímetros cúbicos

CN – controle negativo

LISTA DE SIGLAS

OMS – Organização Mundial da Saúde

PBMC – Células Mononucleares Do Sangue Periférico

DNA – ácido desoxirribonucleico

GLUT 5 – transportador de glicose

ATP – trifosfato de adenosina

SUMÁRIO

1 INTRODUÇÃO

A busca por uma vida saudável, fez com que os hábitos alimentares sofressem modificações. Uma delas foi a substituição de açúcares por adoçantes. De maneira geral, é um modo para prevenir cáries, obesidade e doenças associadas, como diabetes e hiperlipidemia (AHMED *et al*., 2011). No entanto, dados indicam que alguns desses adoçantes têm efeitos prejudiciais à saúde humana (SCHIFFMAN, 2012).

Existe diversos tipos de adoçantes e sua classificação pode ser definida por natural ou artificial, também podendo ser classificados de acordo com sua função em nutritivos e não nutritivos (SWITHERS, 2014). Os adoçantes naturais são substâncias encontrados na natureza com capacidade de aferir sabor doce. Já os adoçantes artificiais são os obtidos em processos químicos equivalentes aos de origem natural. Os adoçantes nutritivos são aqueles que possuem valor calórico e dão gosto adocicado e não nutritivos os que não possuem valor calórico, porém têm doçura acentuada (API et al., 2019).

Os adoçantes permitidos no brasil, pela resolução nº 18, de 24 de março de 2008, são: Acessulfame de Potássio, Aspartame, Ciclamato, Eritritol, Esteviosideos, Isomalte, Lactitol, Maltitol, Manitol, Neotame, Sacarina, Sorbitol, Sucralose, Taumatina e Xilitol (Brasil, 2008).Um estudo de (SYLVETSKY et al., 2017) relatou que nos Estado Unidos, no ano de 1999 de 2012 houve um aumento de 200% no consumo de adoçantes nas crianças acima de dois anos e 54% de aumento entre os adultos.

Existem uma série de questionamentos a respeito da segurança dos adoçantes, podendo oferecer riscos à saúde do consumidor (API et al., 2019). Todavia, ainda faltam muitas informações explicitas sobre os adoçantes.

Considerando a importância que as pessoas atribuem para o uso dos adoçantes, o presente trabalho se propõe analisar os efeitos citotóxicos, genotóxicos e mutagênicos dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni em concentrações semelhantes as utilizadas no consumo humano moderado, utilizando um modelo de células dos Sistema humanos cultivadas em laboratório.

2 CONCEITOS GERAIS E REVISÃO DE LITERATURA

2.1 Aditivos alimentares

A busca por alimentos de baixo valor energético e a procura por adoçantes vem aumentando. Este fato pode estar relacionado aos cuidados com a saúde por causa das doenças que o alto consumo de sacarose provoca, como a obesidade, o diabetes e a cárie dental (HONORATO et al., 2016). Graças a tecnologia industrial, é possível desenvolver produtos saudáveis mantenho o sabor adocicado. Nem sempre a aceitação dos produtos dietéticos é fácil, já que o sabor é dessemelhante e os hábitos terão que ser modificados (CASTRO; FRANCO, 2002). Sendo assim os adoçantes são acrescentados nos alimentos ajudando no sabor e no equilíbrio da ingestão de calorias (HONORATO et al., 2016).

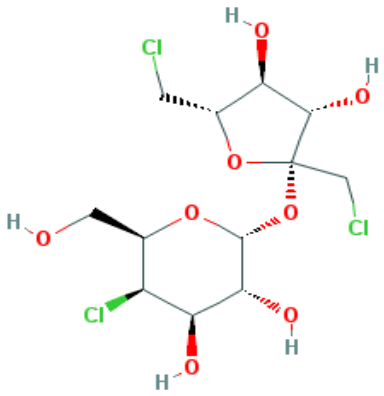
Existem preocupações predominantes sobre a toxicidade desses compostos, já que na maioria dos adoçantes não nutritivos não são metabolizados no organismo. Como mostra o estudo de (ARUN SHARMA, S. AMARNATH, M. THULASIMANI, 2016) que relatam que a ingestão de adoçantes em camundongos por 11 semanas levou ao desenvolvimento de intolerância à glicose, alterando o microbiota intestinal.

2.2 Sucralose

Dentre alguns dos adoçantes mais utilizados, podemos citar a Sucralose (1,6 dicloro–didesoxi-β-D–frutofuranosil–4–cloro–4–deoxi-α-Dgalactopiranosídeo) (Figura 1), um edulcorante organoclorado sintético, obtido a partir da reação de cloração da sacarose (TASHIMA; CARDELLO, 2003).

Apresenta poder adoçante 600 vezes superior à sacarose sendo, portanto, um dos adoçantes de escolha em dietas com restrição calórica. Estudos prévios têm relacionado o uso de Sucralose a vários distúrbios de saúde como aumento dos riscos e incidências de alergias, reações de hiperatividade, ou incidência no desenvolvimento de câncer (HENZ; ARTHUR; COLVARA, 2018). Segundo (SCHIFFMAN; ROTHER, 2013) a Sucralose a altas temperaturas gera cloropropanóis, uma classe potencialmente tóxica de compostos, que poderia estar relacionada aos efeitos citados.

Figura 1 – Estrutura química da Sucralose



Disponível em: <https://pubchem.ncbi.nlm.nih.gov/compound/71485#section=Top>. Acesso em 21 agosto de 2019.

Por outro lado, ensaios *in silico* mostram que a sua estrutura química tem baixa reatividade, nenhum potencial de biotransformação e nenhum alerta em sua estrutura para atividade hemotóxica e cancerígena, sendo também resistente a degradação química e enzimática. Corroborando com as análises computacionais, testes *in vivo*, mostra que a Sucralose não é metabolizada no intestino e quase 85%, excretada intacta. Os demais 15% são metabolizado e biotransformado em conjugados glucuronídeos não tóxicos (BERRY *et al*., 2016).

Outro estudo com camundongos demostrou que a Sucralose prejudicava o DNA gastrointestinal alterando a composição da microbiota. Dados implicam que a microbiota tem um papel nas causas de diversos tipos de câncer, influenciando o processo inflamatório, o dano ao DNA e interferindo no controle do mecanismo de apoptose (SOFFRITTI et al., 2016).

2.3 Frutose

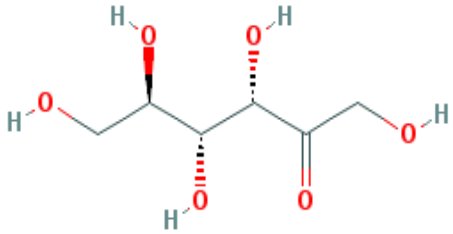
A Frutose (Figura 2) é constituinte da sacarose (β-D-frutofuranosil α-Dglicopiranosida) e de outros polímeros denominados fructanas ou inulina. A Frutose é encontrado em cereais, vegetais e no mel, carrega a propriedade de ser 1,5 vezes mais doce do que a sacarose (GAINO; SILVA, 2011).

O excessivo uso da Frutose como adoçante pela indústria alimentícia, fez com que atingisse uma elevação nos números de casos de síndrome metabólica,

obesidade, hipertensão arterial, *Diabetes mellitus*, resistência à insulina e também casos de esteatose hepática (SIMÕES FERREIRA *et al*., 2018).

A Frutose exerce efeitos característicos sobre os metabolismos lipídico e de carboidratos. Pelo seu potencial bioenergético não compensado, a Frutose pode ser mais prejudicial à saúde. Além de que, vários estudos em humanos sustentam a relação entre o alto consumo de Frutose e a hiperglicemia, resistência à insulina e diabetes tipo 2 (BAENA *et al*., 2016).

Figura 2 – Estrutura química da Frutose



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Acesso em 16 de outubro de 2019.

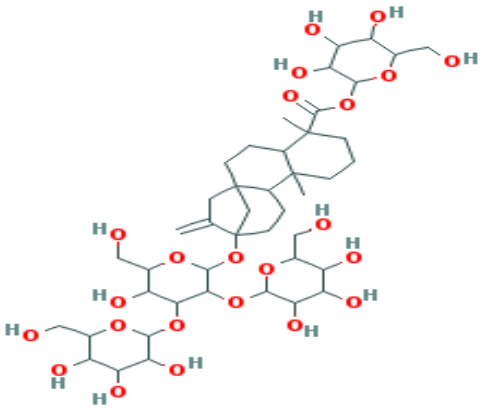
A Frutose é absorvida no intestino e metabolizada pelo fígado humano, através dos transportadores GLUT5, podendo produzir glicose, glicogênio, lactato e piruvato, o que torna um importante substrato de interconversão metabólica (COLLINO, 2011). A ingestão de Frutose também foi associada com maiores níveis de pressão arterial em adolescentes e adultos sem história prévia de hipertensão. Outro estudo aponta que o consumo de Frutose resulta na diminuição da produção dos hormônios leptina e insulina, que estão envolvidos na regulação da homeostase energética e adiposidade corporal. De tal modo, que a longo prazo, o consumo de dietas ricas em e Frutose pode conduzir ao aumento do consumo de energia, ganho de peso e obesidade (CANANI *et al*., 2016).

2.4 *Stevia Rebaudiana* Bertoni e Steviol

Pertencente à família Asteraceae, a *Stevia rebaudiana* Bertoni (Figura 3) tem alto teor de glicosídeos como o rebaudiosídeo A, além de um sabor de 100 a 300 vezes mais doce que a sacarose. Tem sido amplamente usada em muitos tipos de alimentos, medicações, bebidas, cosméticos, na produção de vinho e produtos químicos domésticos (RIZWAN *et al*., 2018).

Por não ser calórico, a *Stevia rebaudiana* Bertoni traz benefícios para a saúde humana e propriedades terapêuticas como: anti-hiperglicêmicas, anti-hipertensivas, anti-oxidantes, antitumorais, antidiarreicas, diuréticas, gastroprotetoras e imunomoduladoras (PRATA et al., 2017)

Figura 3 – Estrutura química do Esteviosídeo, princípio ativo da *Stevia rebaudiana* Bertoni



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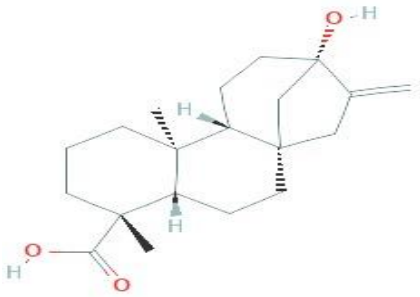
Acesso em 16 de outubro 2019.

Um estudo realizado durante 90 dias em ratos com administração de extrato de *Stevia rebaudiana* Bertoni não mostrou indícios de toxicidade significativos, assim como no teste de micronúcleo (ZHANG et al., 2017).

Da mesma forma, (WILLIAMS; BURDOCK, 2009) não observaram sinais de citotoxicidade em ratos Wistar que receberam Rebaudioside A nas concentrações de 150, 375 ou 750 mg/kg. Já o Steviol (Figura 4) é um glicosídeos diterpenóide natural isolados das folhas de *Stevia rebaudiana* Bertoni, amplamente utilizado como um adoçante não calórico e principal metabólito ativo da *Stevia rebaudiana*, já que os

Steviosídeos são convertidos a Steviol segundo após sua ingestão (CHRISTINE et al., 2019)

Figura 4 – Estrutura química do Steviol



Disponível em https://pubchem.ncbi.nlm.nih.gov/compound/452967#section=2DStructure. Acesso em 16 de outubro de 2019.

Segundo a Organização Mundial de Saúde (OMS), a partir de 2011 as indústrias foram autorizadas a utilizar o Steviol, após a sua segurança ser avaliada e uma ingestão diária ser estabelecida (PANAGIOTOU et al, 2018).

Além do seu gosto adocicado, os glicosídeos de Steviol também podem exercer benefício terapêutico, pois possui comprovada ação anti-inflamatória, antihiperglicêmica, anti-hipertensiva, antitumoral, antidiarreica, diurética e imunomoduladora (PANAGIOTOU et al, 2018).

Até o momento, foram encontrados poucos relatos sobre a citotoxicidade do Steviol em células humanas. Segundo (CHEN et al., 2018) há relato de que em células U2OS (linhagem de Osteosarcoma), o Steviol apresenta atividade anticancerígena.

2.5 Sacarina

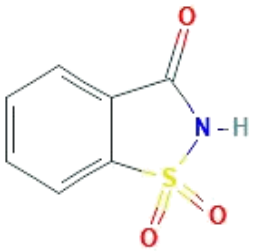
Descoberta acidentalmente em 1879 por Ira Remsen e Constantine Fahlberg, a Sacarina é adoçante artificial mais antigo em uso (Figura 5) (AHMED et al., 2011).

A Sacarina apresenta poder edulcorante 200 a 700 vezes superior ao da sacarose, elevada estabilidade, não é higroscópica e tem poder calórico nulo. Além

disso, não é metabolizado no sistema digestivo humano e é excretado rapidamente pela urina, não sendo acumulando no organismo humano (BARREIROS, 2012) .

No entanto, tem sabor amargo e metálico, o que faz a Sacarina ser muitas vezes utilizada em combinação com outros adoçantes (SWITHERS, 2014).
(BARREIROS, 2012).

Figura 5 – Estrutura química da Sacarina



Disponível em https://pubchem.ncbi.nlm.nih.gov/compound/5143#section=2DStructure. Acesso em 16 de outubro de 2019.

Seu passado é controverso. Estudos demostraram que a Sacarina era carcinogênica em ratos, por essa razão ela era vendida, mas com advertências. Já estudos subsequentes demonstraram que a Sacarina não é tóxica nem carcinogênica em quantidades normais, pois ela seria cancerígena somente em animais e isso não se aplicava em humanos (AHMED et al., 2011).

Segundo (CELESTE; ANDRADE, 2011), em um estudo empregando ratos gestantes pode notar que a Sacarina cruza a barreira placentária e ainda é identificada no sangue do cordão umbilical logo após o parto. Por esse motivo, deve-se restringir o uso de Sacarina na gestação e na lactação (BRUGNERA; BARUFFI; PANATTO, 2012).

2.6 Sistema Imune

O sistema imune é muito importante para a compreensão e o funcionamento dos processos fisiológicos, ele permite ao organismo reconhecer-se e assim faz com que promova respostas apenas ao que é estranho (CALICH; VAZ, 2009). Assim, quando o corpo humano é exposto a determinados agentes estranhos como bactérias,

parasitas, fungos e vírus, o sistema imunológico desencadeia uma série de eventos com o propósito de eliminar esses agentes, caracterizando dessa forma uma resposta imune (VAZ; TAKEI; BUENO, 2010) e diferentemente de outros sistemas, as células que o compõe são móveis o que facilita a identificação de qualquer molécula, microrganismos estranhos e alterações em células próprias (BALESTIERI, 2006).

Os eventos provocados pelo sistema imune podem ser denominados de resposta imunológica, na qual se divide em resposta inata ou resposta adaptativa. A resposta inata possui barreiras físicas, químicas e a participação de outras células (macrófagos, neutrófilos, células dendríticas e células natural killer). A resposta adaptativa envolve principalmente linfócitos T, B e os seus produtos, citocinas e anticorpos, respectivamente. Pode ser dividido em uma resposta imune humoral (mediada por anticorpos) e uma resposta imune celular (mediada por células, como linfócitos T e macrófagos) (TERRA et al., 2012).

As células do sistema imune são originadas pelas células hematopoiéticas que são as responsáveis por atribuírem a ele suas principais características, especificidade e memória. Assim as células que fazem parte desse sistema são: os linfócitos T, linfócitos B e células natural killer (NK), as células apresentadoras de antígeno (macrófago, células dendríticas, células de Langerhans e os próprios linfócitos B), e os órgãos que o compõe são classificados como órgãos linfoides primários (timo e a medula óssea) os quais são responsáveis pela geração de linfócitos e os órgãos linfoides secundários (baço, linfonodos, tecido linfoide associado às mucosas do aparelho respiratório) (LEANDRO et al., 2002; CALICH; VAZ, 2009).

2.6.1 Linfócitos

Existem dois tipos de linfócitos, o T e o B, ambos são produzidos na medula óssea e os responsáveis pelo reconhecimento específico de antígenos por meio de proteínas presentes em suas superfícies (VAZ; TAKEI; BUENO, 2010).

Os linfócitos B são inicialmente produzidos no saco vitelino, na vida fetal a produção passa a ser no fígado e por último na medula óssea, onde serão diferenciados como efetores, células produtoras de anticorpos ou de memória (BALESTIERI, 2006; KINDT; GOLDSBY; OSBORNE, 2008; VAZ; TAKEI; BUENO, 2010).

A ativação de linfócitos B é caracterizada como uma resposta humoral, mediada por imunoglobulinas com função de anticorpo os quais podem neutralizar ou destruir os antígenos contra os quais foram gerados (VAZ; TAKEI; BUENO, 2010), de modo que a porção humoral do sistema imune age na interação das células B com o antígeno e consequentemente na proliferação e diferenciação nas células plasmáticas secretoras de anticorpos (glicoproteínas), onde em suas extremidades aminoterminais é formado o sítio de ligação do antígeno (KINDT; GOLDSBY; OSBORNE. 2008).

Os linfócitos T passam da medula óssea para o timo, se diferenciam em linfócitos T e podem ser divididos em linfócitos T auxiliadores (ativa outras células para 21 exercerem suas funções) ou linfócitos T citotóxicos (possuem a função de eliminar células tumorais e/ou infectadas, porém dependem dos linfócitos T auxiliadores) (BALESTIERI, 2006).

As células T auxiliadoras são a chave da resposta imune e a ativação de outras células depende de sua prévia ativação. Porém, são capazes de reconhecer moléculas próprias associadas com estranhas e possuem mecanismos de proteção para que não ocorra a estimulação dessas células de forma exagerada, o que ao contrário iria gerar uma doença auto-imune (BALESTIERI, 2006).

3 OBJETIVOS

3.1 Objetivo Geral

- Avaliar as concentrações citotóxica, genotóxicas e mutagênicas dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni.

3.2 Objetivos Específicos

- Determinar os efeitos dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni sobre parâmetros citotóxicos (viabilidade e proliferação celular) em culturas de linfócitos humanos e de suas subpopulações.

- Determinar os efeitos dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni sobre parâmetros genotoxicológicos (teste cometa) em culturas de linfócitos humanos;

- Determinar os efeitos dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni sobre parâmetros mutagênicos (instabilidade cromossômica) em culturas de linfócitos humanos;

- Determinar predições de possíveis de mecanismo de ação dos adoçantes Sacarina, Sucralose, Frutose e *Stevia rebaudiana* Bertoni utilizando plataformas computacionais.

4 METODOLOGIA

4.1 Obtenção dos adoçantes

Os adoçantes Sacarina (CAS n. 81-07-2) Sucralose (CAS n. 56038-13-2) Steviol (CAS n. 471-80-7) e Frutose (CAS n. 57-48-7) foram obtidos comercialmente de empresa Sigma-Aldrich®.

4.2 Preparo da Cultura de Linfócitos Humanos

As culturas de linfócitos foram preparadas utilizando sangue venoso obtido por venopunção de voluntário autointitulado saudável (CEP UNIPAMPA 27045614.0.0000.5323) e transferido para tubos falcon contendo o reagente de separação Histopaque-1077® (1:1). Em seguida, a suspensão foi centrifugada por 30 min a 400 x g e a interface opaca contendo as PBMC cuidadosamente foi aspirada e transferida para um tubo falcon limpo. As células foram lavadas adicionando meio RPMI 1640 e centrifugadas a 250 x g durante 10 min. O sedimento foi ressuspenso com meio RPMI 1640. A suspensão de PBMC foi mantida por 24 horas em estufa a 37ºC em ambiente de 5% de CO_2, em meio de cultura RPMI 1640 suplementado com 10% de soro bovino fetal, penicilina (100 U/mL) e estreptomicina (100 mg/mL), conforme descrito em trabalho prévio do nosso grupo (DUARTE et al., 2018). Após esta pré-incubação, os linfócitos foram separados dos monócitos que ficaram aderidos a superfície de contato. Os linfócitos foram estimulados pela adição de fitohemaglutinina-M a 1 mg/mL para experimentos subsequentes. A densidade celular foi ajustada para cada protocolo e todas as culturas e análises foram realizadas em triplicata.

4.3 Curva-efeito para avaliação da toxicidade

As culturas foram preparadas de acordo com o protocolo descrito no item 4.2. As concentrações utilizadas abrangeram um amplo espectro, buscando avaliar a maior faixa de utilização possível. As células foram expostas a concentrações de 1 µg / mL, 10 µg / mL, 50 µg / mL, 100 µg / mL, 250 µg / mL e 500 µg / mL. Os parâmetros

analisados foram a contagem e a viabilidade celular, analisadas através da perda da integridade da membrana, utilizando o método do Azul de Tripan (BUROW et al., 1998). Após estes testes, foi calculada a Concentração Letal 50 (CL_{50}), concentração onde 50% dos linfócitos estavam mortos, utilizando como referência para cálculo das doses para os demais testes.

4.4 Análise de parâmetros genotoxicológicos

Para estes testes foi utilizada a cultura da forma descrita no item 4.2. Os parâmetros avaliados foi: Dano de DNA, pelo Teste Cometa (SINGH, 1988) e Instabilidade Cromossômica, numérica e estrutural, avaliada pela técnica de Citogenética Clássica descrita por Yunis (1976).

4.5 Efeitos dos adoçantes em subpopulações linfocitárias

Os linfócitos foram suspensos em meio a uma densidade de 0,5 x 10^6 / por poço da placa de 12 poços e expostos aos adoçantes: Sacarina, Sucralose, Frutose e Steviol, em 3 diferentes concentrações determinadas pela curva-efeito por 24 h. A toxicidade específica para subpopulações de linfócitos CD3, CD4 e CD8 foi avaliada pelo Contador Automático de Células Countess II FL (Thermo Fisher, Massachusetts, EUA) equipado com cubo de fluorescência EVOS™ Light Cube GFP e adição de Anti-CD3 (FICT), AntiCD4 (FITC) e Anti-CD8 (FITC) previamente nas amostras de acordo com as especificações do fabricante (Abcam, Cambridge, MA).

4.6 Análise *In silico* dos adoçantes

De forma complementar e buscando possíveis métodos de ação em humanos, adoçantes foram submetidos a uma série de testes computacionais (*In Silico*) através das plataformas: PASS On-Line (*Prediction of Activity Spectra for Substances*) no módulo DIGEP (*Drug-induced gene expression profile*) e GeneCards. Foram analisados os efeitos em 14.700 genes e relacionados aqueles com predição superior a 70% e relacionados a aspectos sobrevida ou genética celular. Os endereços dessas plataformas estão nas referências.

4.7 Análise Estatística

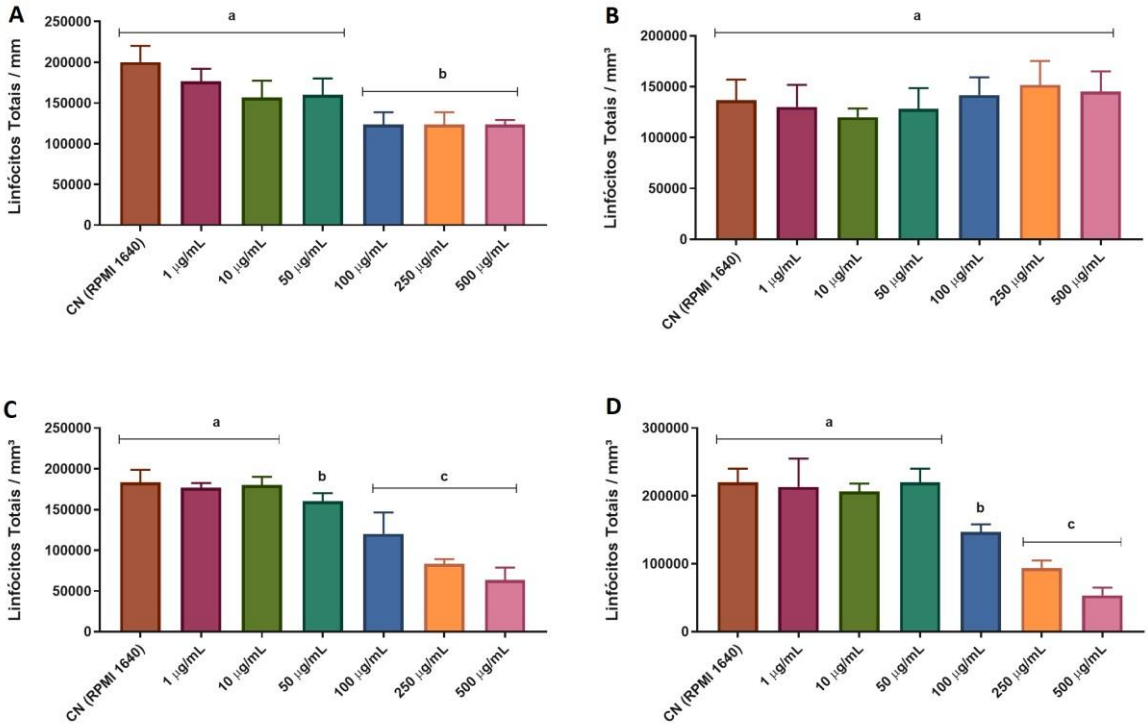
Os dados foram expressos como média ± desvio padrão. Todas as análises foram realizadas em software estatístico específico. Para a curva de citotoxicidade foi utilizada regressão não linear; para as demais análises foi empregada análise de variância de uma via (ANOVA) seguida de teste *Post-Hoc* de Tukey. Foram considerados significativos os resultados com valor de $p<0,05$.

5 APRESENTAÇÃO DA PESQUISA E ANÁLISE DOS RESULTADOS

5.1 Curva-Efeito para Avaliação da Toxicidade

A Figura 06 representa a curva-efeito para a avaliação da citotoxicidade da Sucralose (A), Frutose (B), Steviol (C) e Sacarina (D) em linfócitos. Foram utilizadas faixas amplas de concentrações variando de 1 µg/mL até 500 µg/mL para se obter a CL_{50}. Utilizando o cálculo de regressão não-linear (as *curvas não são mostradas aqui*), a CL_{50} obtida para cada adoçante foi de 78,51 µg/mL para a Sucralose, superior a 500 µg/mL para a Frutose, 178,7 µg/mL para Steviol e 321,2 µg/mL para Sacarina. A partir deste resultado, determinamos as concentrações que seriam testadas nos experimentos seguintes para análise de parâmetros citotóxicos, mutagênicos e genotoxicológicos comparativos, sendo de relevância obter concentrações que permitam uma viabilidade celular sempre superior a 80% (RICEEVANS, MILLER, PAGANGA, 2006). Assim as concentrações-teste dos experimentos foram definidas como 1 µg/mL, 10 µg/mL e 50 µg/mL para todos os adoçantes.

Figura 06: Avaliação da proliferação celular para determinar o LC_{50} dos adoçantes em cultura de linfócitos.

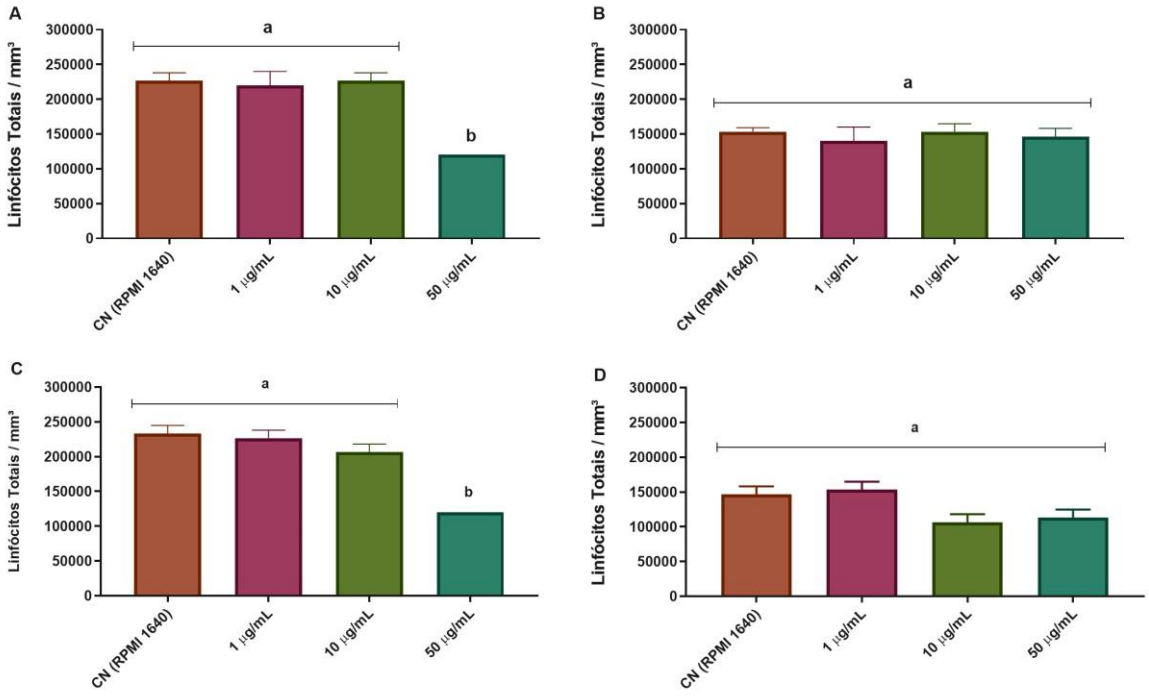


Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. CL_{50} foram calculadas utilizando regressão não-linear.

5.2 Análise de parâmetros citotóxicos

A Figura 07 representa a proliferação celular. Nota-se que a Sucralose e o Steviol tiveram uma diminuição no número de linfócitos na concentração de 50 µg/mL na ordem de aproximadamente 48% nos dois casos. Já, os adoçantes Frutose e Sacarina não afetaram a proliferação nas concentrações testadas.

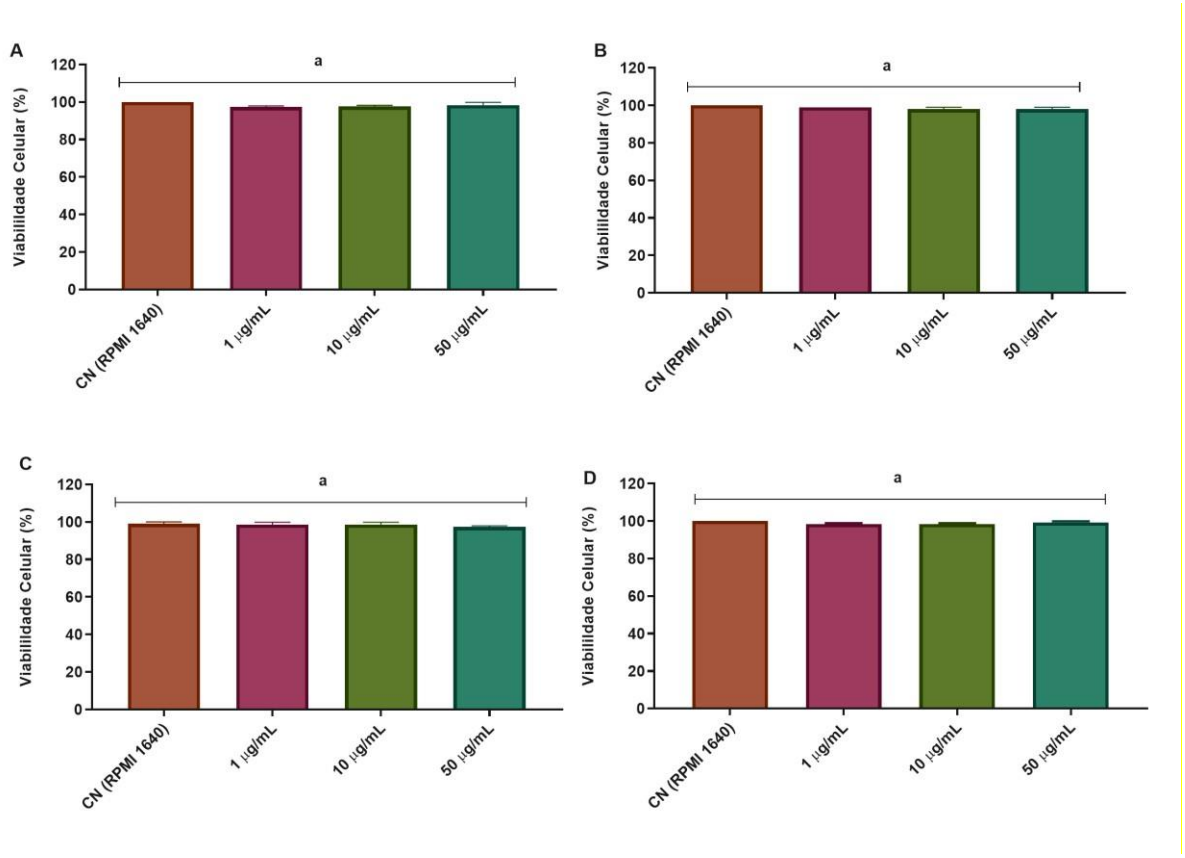
Figura 07: Avaliação da proliferação celular de linfócitos humanos expostos a diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

A Figura 08 corresponde à viabilidade celular, observou-se que mesmo na maior concentração dos adoçantes testada, 50 µg/mL, não houve diminuição de número de linfócitos comparado com o controle negativo, o que condiz com os resultados mostrados previamente na curva de toxicidade.

Figura 08: Avaliação da viabilidade celular de linfócitos humanos expostos a diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

As figuras 9, 10 e 11 apresentam os efeitos dos adoçantes Sucralose, Frutose, Steviol e Sacarina, em subpopulações linfocitárias $CD3^+$, $CD4^+$ e $CD8^+$, respectivamente.

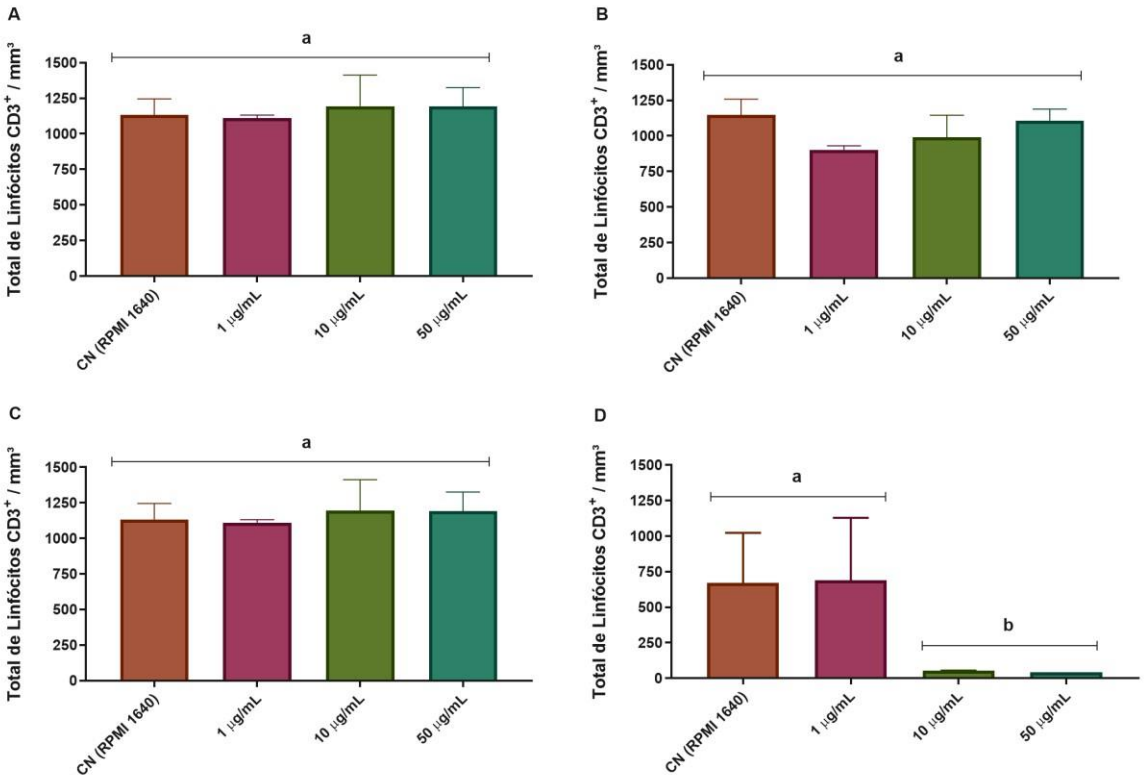
Observou-se que a Sacarina apresentou uma inibição de linfócitos T $CD3^+$ nas concentrações 10 µg/mL e 50 µg/mL. Estas diminuições foram significativas e alcançaram valores em torno de 91% e 93%, respectivamente.

Já em relação as quantidades de linfócitos T $CD4^+$, averiguou-se que Steviol, Sucralose e Frutose proporcionaram uma diminuição nas concentrações 10 µg/mL e 50 µg/mL. Estas reduções variaram entre 35% e 70% dependendo das concentrações

e do adoçante, mas são reduções expressivas e significativas. No entanto a Sacarina não obteve resultado significativo em nenhuma das concentrações testadas.

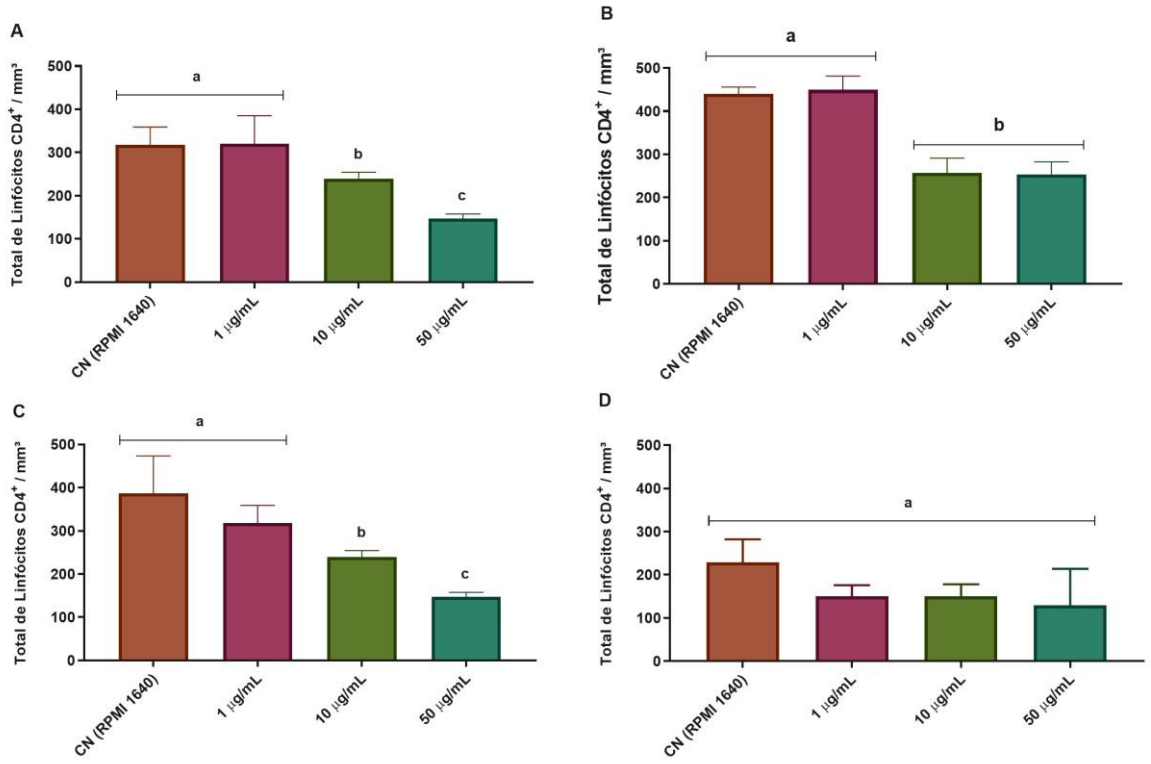
Na subpopulação de linfócitos de T $CD8^+$, os adoçantes Sucralose e Steviol mostraram uma depleção celular em todas as concentrações testadas, variando de 18% a 62%. Já os Sacarina e Frutose, causaram uma diminuição de linfócitos T CD8+, nas concentrações 10 µg/mL e 50 µg/mL, variando de 32% a 94%.

Figura 09: Citotoxicidade em Subpopulações CD3+ de linfócitos humanos expostos a diferentes adoçantes em cultura celular.



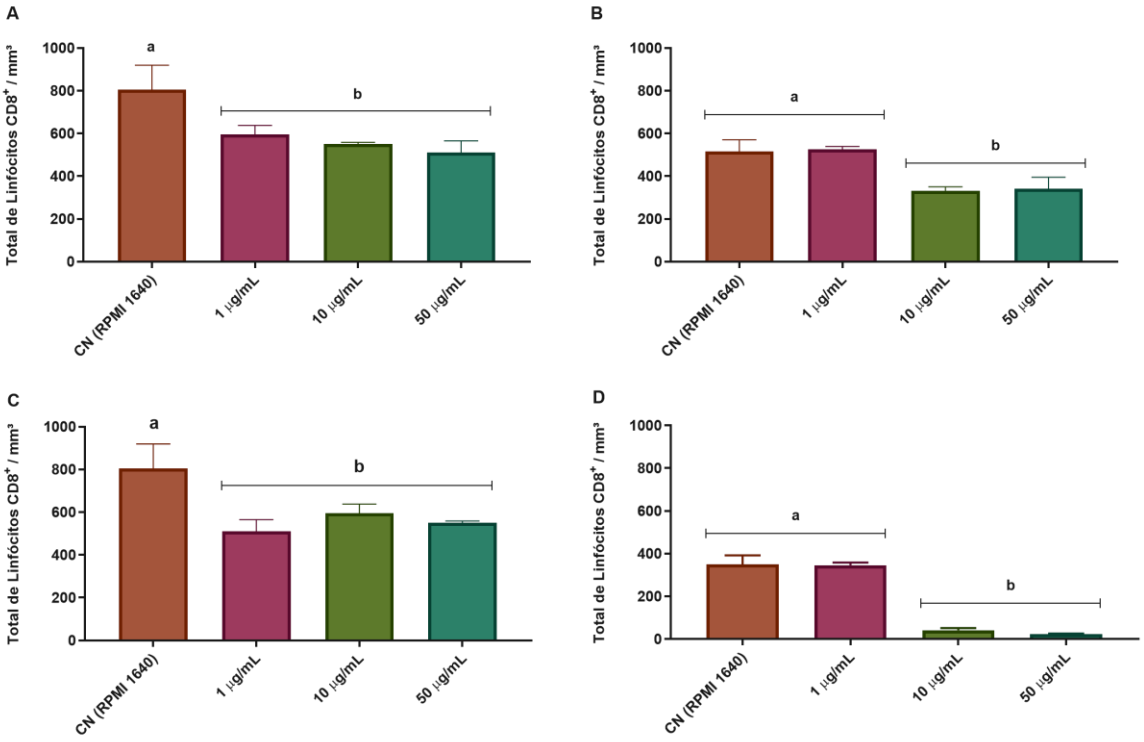
Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

Figura 10: Citotoxicidade em Subpopulações CD4+ de linfócitos humanos expostos a diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

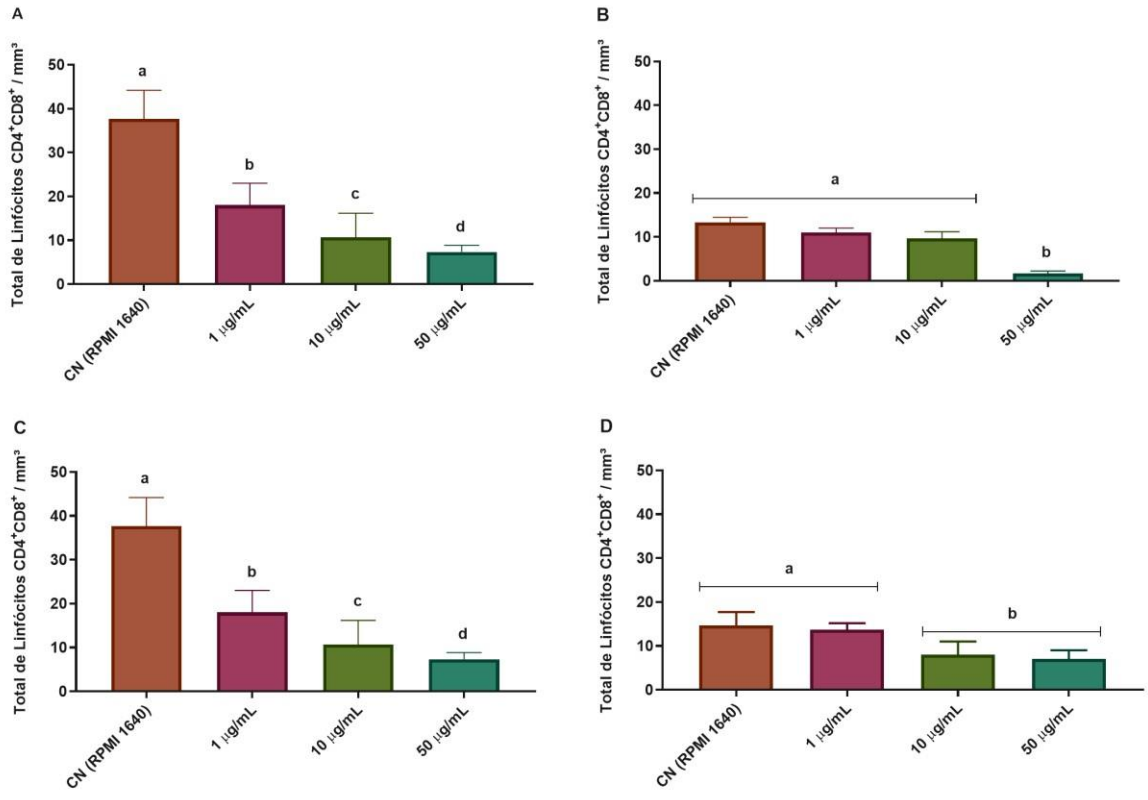
Figura 11: Citotoxicidade em Subpopulações CD8+ de linfócitos humanos expostos a diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados estão apresentados como média ± desvio padrão, e foram realizados em triplicata. Letras diferentes representam valores diferentes para $p<0,05$.

A figura 12 apresenta efeitos dos adoçantes Sucralose, Frutose Steviol e Sacarina em dupla população de linfócitos cultivados. Os linfócitos Dupla População são considerados células jovens, imaturas e que ainda não terminaram seu processo de amadurecimento celular (ZUCKERMANN; GASKINS, 1996). Identificou-se que na Sucralose e no Steviol, os linfócitos dupla-população, apresentaram diminuição celular em todas as concentrações testadas. Já com a Sacarina, constatou-se uma inibição celular apenas nas concentrações de 10 µg/mL e 50 µg/mL. Na Frutose a diminuição de linfócitos acontece apenas na concentração de 50 µg/mL na ordem de 87%.

Figura 12: Citotoxicidade em Subpopulações Dupla População ($CD4^+CD8^+$) de linfócitos humanos imaturos expostos a diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

As figuras 06 a 12 mostram resultados de parâmetros de citotoxicidades. Em com concentrações diferentes ou parâmetros diferentes, podemos observar uma mesma conclusão: todos os adoçantes testados apresentam algum grau de linfotoxicidade e em alguns casos específica a alguma subpopulação celular.

Um estudo de (BESSLER; DJALDETTI, 2019) vai de encontro aos nossos dados. Neste trabalho, foram utilizados Sacarina, Sucralose e Stevia, ambos comerciais, na concentração de 10 µg/mL em cultura de PBMC e células de câncer de colón, pelo ensaio XTT não apresentando efeito sobre a viabilidade celular.

Outros parâmetros não foram testados.

Segundo o trabalho de (VAN EYK, 2015) constatou que os adoçantes Sucralose e Sacarina na concentração de 10mM (equivalente a 4000µg/mL para Sucralose e 2000µg/mL para Sacarina), nas células Caco – 2 e HT-29 (linhagem celular de colón) obtiveram uma diminuição da viabilidade celular pelo ensaio de MTT e não obteve diminuição celular em HEK - 293 (linhagem celular de rim).

Já para o resultado do Steviol um achado demonstra que em 6 tipos de células gastrointestinais diferentes, o mesmo mostra citotoxicidade pelo ensaio de MTT, porém em concentração de 100 a 200 µg/mL (CHEN et al., 2018).

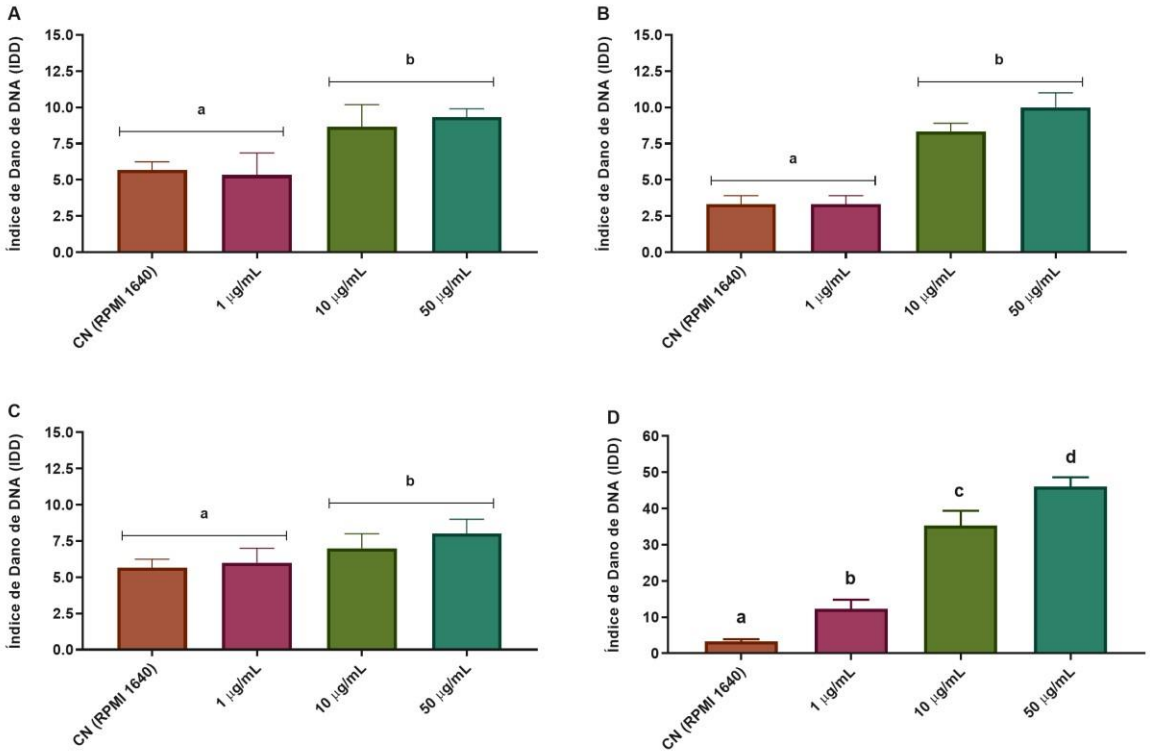
5.3 Análise de parâmetros genotoxicológicos

Na Figura 13 estão representados os índices de dano de DNA causados sob efeitos dos adoçantes.

O adoçante Sacarina apresentou um dano em todas as concentrações testadas, variando de um aumento de 2,5x na menor concentração até um aumento de 13x na maior concentração testada. Os demais adoçantes, mostraram um dano médio de aproximadamente 62% maior do que o grupo controle negativo nas concentrações 10 µg/mL e 50 µg/mL, sem diferença estatística entre as concentrações.

Novamente outros pesquisadores reforçam nossos resultados. (VAN EYK, 2015) constatou que a Sacarina e Sucralose apresentaram dano de DNA pelo ensaio cometa alcalino em uma cultura celular de cólon (Caco-2 e HT-29) e rim de (HEK-293) nas concentrações de 0,1mM, 1,0mM e 10mM.

Figura 13: Avaliação dos Danos de DNA celular de linfócitos humanos causados por diferentes adoçantes em cultura celular.



Legenda: Em (A) Sucralose, em (B) Frutose, em (C) Steviol e em (D) Sacarina. Os dados são expressos como média ± desvio padrão, realizados em triplicatas. Letras diferentes representam valores diferentes para $p<0,05$.

5.4 Análise de parâmetros mutagênicos

Por sua vez, a Tabela 02 e a Figura 14 mostram os dados do teste de Instabilidade Cromossômica. Neste teste são contadas 300 células em metáfase, sendo avaliadas alterações cromossômicas numéricas e estruturais como: anelamento, cariopicnose, chanfradura, quebra, ligamento etc.

Na Sucralose todas as concentrações (1, 10 e 50 µg/mL) causaram alterações estruturais) proporcionalmente a concentração. O tipo de alteração estrutural observado em todos os casos foi a *Cariopicnose*.

Nos adoçantes Steviol e Sacarina observou-se alterações nas maiores concentrações, 10 e 50 µg/mL. O tipo de alteração estrutural observado foi a *Cariopicnose* e a Fusão Centromérica.

Na Frutose nenhuma das concentrações causou alteração nos parâmetros analisados.

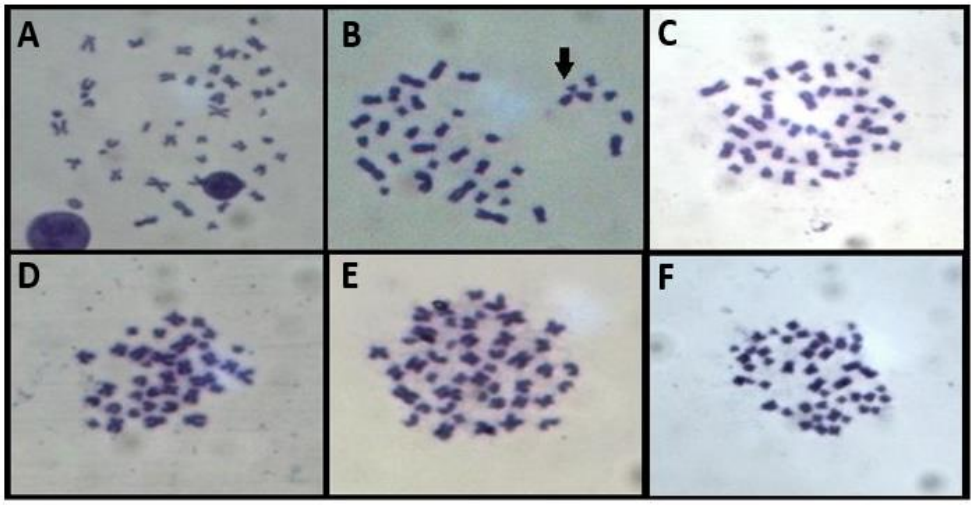
Tabela 01: Aberrações cromossômicas numéricas e estruturais induzidas pelos diferentes em linfócitos humanos cultivados.

	Grupo	Índice Mitótico (Média ± DP)	Metáfases totais contadas	Metáfases Normais	Alterações numéricas	Alterações estruturais	Tipo de alteração encontrada
FRUTOSE	Controle Negativo	1,40 ± 0.2[a]	300	299 (Figura 14A)	01	00	----------
	1 µg/mL	1,30 ± 0,1[a]	300	300	00	00	----------
	10 µg/mL	1,40 ± 0,2[a]	300	299	01	00	----------
	50 µg/mL	1,40 ± 0,1[a]	300	299	01	00	----------
SACARINA	Controle Negativo	1,40 ± 0.2[a]	300	299	01	00	----------
	1 µg/mL	0,80 ± 0,1[a]	300	300	00	00	----------
	10 µg/mL	0,7 ± 0,2[a]	300	241	00	59	*Fusão Centromérica* (Figura 14B)
	50 µg/mL	0,5 ± 0,1[a]	300	298	00	102	*Cariopicnose* (Figura 14C)
STEVIOL	Controle Negativo	1,40 ± 0.2[a]	300	299	01	00	----------
	1 µg/mL	1,30 ± 0,1[a]	300	300	00	00	----------
	10 µg/mL	1,20 ± 0,1[a]	300	251	00	49	*Cariopicnose*
	50 µg/mL	1,10 ± 0,1[a]	300	213	00	87	*Cariopicnose* (Figura 14D)
SUCRALOSE	Controle Negativo	1,40 ± 0.2[a]	300	299	01	00	----------
	1 µg/mL	1,30 ± 0,1[a]	300	262	00	38	*Cariopicnose* (Figura 14E)
	10 µg/mL	1,30 ± 0,1[a]	300	236	00	64	*Cariopicnose* (Figura 14F)
	50 µg/mL	1,30 ± 0,1[a]	300	188	00	112	*Cariopicnose*

Os dados são expressos como média ± desvio padrão, realizados em triplicatas.

Letras diferentes representam valores diferentes para $p<0,05$.

Figura 14: Aberrações cromossômicas induzidas pelos diferentes em linfócitos humanos cultivados.



Legenda: Foto de microscopia óptica em 400X. De A a F são mostrados exemplos de conjuntos cromossômicos normais (A) e com alterações estruturais (B a F).

Outros trabalhos no já realizaram o ensaio de aberração cromossômica em linfócitos humanos com Stevia e observaram alterações estruturais (quebras) nas concentrações de 1 a 16 µg/ml (UÇAR et al., 2018).

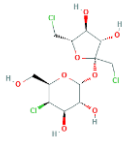
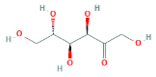
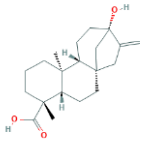
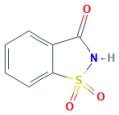
Já um estudo de mutagenicidade pelo teste de Ames (BANDYOPADHYAY; GHOSHAL; MUKHERJEE, 2008) demonstrou que a Sacarina foi negativo nas concentrações de 50, 100 e 200 µg/placa, em um teste (SHASTRY C.S., YATHEESH C.K., 2012) utilizando concentrações de ingestão diária aceitável de Sucralose em ratos por três semanas mostrou que no teste de Ames não resultou efeito mutagênico.

Também, um estudo demostrou que uma sobrecarga de sacarose (concentrações de 6,9%, 13,8% ou 34,8% (p/p) na alimentação de ratos, mostrou uma mutação nas células do colón nas maiores concentrações, mas não nas células do fígado (DRAGSTED et al., 2002).

5.5 Avaliação *In Silico* de interações gênicas dos adoçantes

De forma complementar, foram analisadas as interações com genes relacionados ao ciclo celular ou com aspectos genéticos da célula. Os resultados estão compilados na **Tabela 02**.

Tabela 02: Predição dos efeitos dos adoçantes sobre genes envolvidos com sobrevida e genética celular.

Gene	Sucralose		Frutose		Steviol		Sacarina		Efeito biológico da proteína codificada pelo gene
									
	Efeito	**%***	**Efeito**	**%***	**Efeito**	**%***	**Efeito**	**%***	
MAPK8	↓	90,9	↓	91,6	n.a				Envolvido com proliferação e diferenciação celular.
APTX	↓	89,6	↓	90,5	n.a				Relacionado a reparo de DNA.
EID1	↑	83,2	n.a						Envolvido com regulação de Histonas.
TFAM	n.a						↓	77,8	Envolvida no reparo do DNA.
SIVA1	n.a						↓	96,1	Gene envolvido da regulação do ciclo celular.
TUBGCP3	n.a		↓	82,9	n.a				Gene envolvido da regulação do ciclo celular.
LST1	n.a		↑	86,4	n.a				Gene que inibe a proliferação de linfócitos.
SESN1	n.a				↓	93,2	n.a		desempenham um papel na resposta celular a danos no DNA.
NAP1L1	n.a				↓	87,5	n.a		Participa da replicação do DNA, modulação da cromatina e regulação da proliferação celular.
SOX4	n.a				↓	81,2	n.a		Envolvida no processo de Apoptose.
TREX1	n.a				↓	78,9	n.a		Envolvida no processo de reparo de DNA.

Legenda: ↓ = reduz a expressão da proteína codificada pelo gene; ↑ = aumento da expressão da proteína codificada pelo gene; * porcentagem de chance de o composto causar a interferência prevista; **n.a. = não se aplica.

Apesar de não termos na literatura um mecanismo relatado para estes compostos causar estes efeitos aqui demostrados, uma possível alternativa é descrita pela avaliação *In Silico*. De acordo com estes testes, como podemos ver na tabela acima, o principal mecanismo envolvido com o dano ou alterações no DNA está relacionado com a modulação da expressão gênica, interferindo em proteínas relacionados à replicação, manutenção e reparo de dano ao DNA.

6 CONCLUSÃO

Pode se concluir que os adoçantes Sacarina, Sucralose, Frutose *e Stevia rebaudiana* Bertoni apresenta efeitos citotóxicos, genotóxicos e mutagênicos nas concentrações e condições testadas em cultura celular de linfócitos humano.

O provável mecanismo parece estar relacionado com a modulação da expressão gênica, interferindo em genes / proteínas relacionadas à replicação, manutenção e reparo de dano ao DNA.

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ANEXOS I

MANUSCRITO(S) SUBMETIDOS OU EM FASE DE REDAÇÃO REFERENTES A ESTA DISSERTAÇÃO

MANUSCRITO I

SUCRALOSE CAUSES NON-SELECTIVE CD4 AND CD8 LYMPHOTOXICITY VIA PROBABLE REGULATION OF THE MAPK8 / APTX / EID1 GENES: AN *IN VITRO* / *IN SILICO* STUDY.

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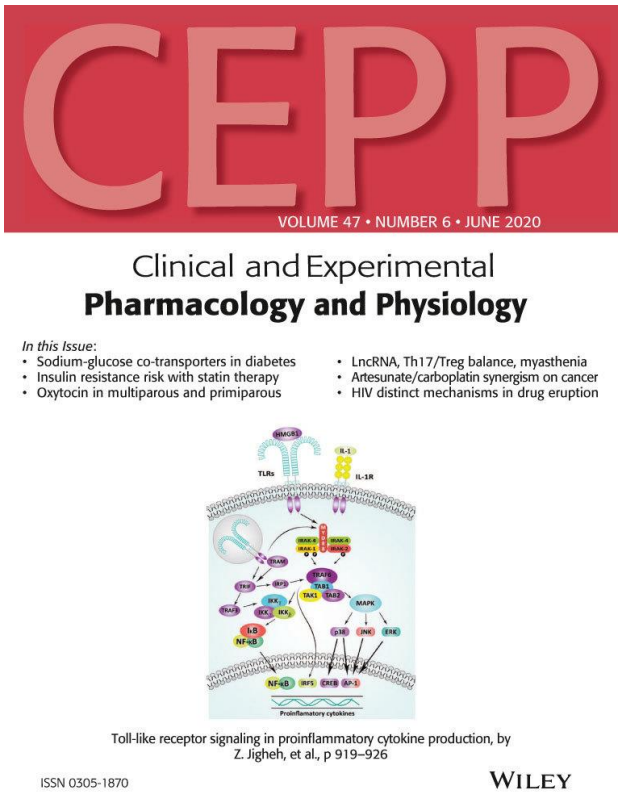

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SUCRALOSE CAUSES NON-SELECTIVE CD4 AND CD8 LYMPHOTOXICITY VIA PROBABLE REGULATION OF THE MAPK8 / APTX / EID1 GENES: AN *IN VITRO* / *IN SILICO* STUDY.

Thaís Pasqualli [1,2], Pamella Eduardha Espindola Chaves[1], Lavínia da Veiga Pereira[1], Élvio Adílio Serpa[1,2], Luís Flávio Souza de Oliveira [1,2], Michel Mansur Machado [1,2,*]

[1] TOXCEL - Grupo de Pesquisa em Toxicologia Celular, Universidade Federal do Pampa, Uruguaiana, Brasil;

[2] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Pampa, Uruguaiana, Brasil.

E-mail address:

Thaís Pasqualli thais.pasqualli@yahoo.com.br

Pamella Eduardha Espindola Chaves pamella.eduardha.chaves@gmail.com

Lavínia da Veiga Pereira lavinia-veiga@hotmail.com

Élvio Adílio Serpa serpaelvio@gmail.com

Luís Flávio Souza de Oliveira luisoliveira@unipampa.edu.br

Michel Mansur Machado michelmachado@unipampa.edu.br

* Send correspondence to: Prof. Dr. Michel Mansur Machado, Universidade Federal do Pampa – Campus Uruguaiana, BR 472, Km 585, Uruguaiana, RS, Brasil, CEP: 97.500970. Tel / Fax: 55 - 55 39110200

E-mail address: michelmachado@unipampa.edu.br

ABSTRACT

One of the most used sweeteners in the world is Sucralose. With sweetening power 600 times greater than sucrose, its use grows among those who seek to cut calories. Research shows that when heated, sucralose generates toxic products that attack the organism and interact with DNA. Our objective was to test this sweetener under unheated conditions and at average concentrations of consumption, evaluating parameters of cytotoxicity, genotoxicity, and immunotoxicity. For this purpose, we made use of lymphocyte cultures and the analysis of their $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations. In a complementary way, the mechanism of action is proposed here by computational methods. Our results showed that sucralose reduces non-selectively the total lymphocytes due to falls in the levels of the $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subpopulation. Besides, we observed an increase in the level of DNA damage and a gradual incidence of structural changes in the lymphocyte chromosomal sets. It was possible to propose that sucralose modulate the gene expression, interfering, especially the MAPK8, APTX, and EID1 genes. Thus, this article presents the results of an evidence-based approach to the safety of human health in the use of sucralose.

Keywords: *Sucralose; In vitro; In silico; gene regulation; lymphocytes; CD8; CD4.*

INTRODUCTION

As one of the most using most sweeteners, we can mention sucralose (1,6 dichloro–didesoxi-β-D–frutofuranosil–4–chloro–4–deoxy-α-galactopiranosídeo (Figure 1), a synthetic organochlorine sweetener, obtained from the chlorination reaction of sucrose (1).

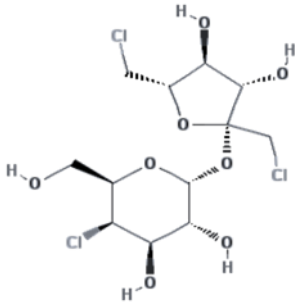

Figure 1 - Chemical structure of Sucralose.

It has a sweetening power 600 times higher than sucrose and is, therefore, one of the sweeteners of choice in diets with calorie restriction. Previous studies have linked the use of sucralose to various health disorders like increased risks and incidences of allergies, hyperactivity reactions, or incidence in the development of cancer. Other authors (2, 3) claim that sucralose at high temperatures generates chloropropanols, a potentially toxic class of compounds, which could be related to the effects mentioned.

On the other hand, *in silico* tests show that its chemical structure has low reactivity, no potential for biotransformation, and no alert in its structure for hemotoxic and carcinogenic activity, it has also been resistant to chemical and enzymatic degradation. Corroborating with computational analysis, *in vivo* tests, shows that the intestine does not metabolize sucralose, and we excrete almost 85% intact. Our organism will metabolize the remaining 15% and biotransform into non-toxic glucuronide conjugates (4).

Another study with mice showed that sucralose harmed gastrointestinal DNA by altering the composition of the microbiota. Data imply that the microbiota has a role in the causes of several types of câncer, influencing the inflammatory process, DNA damage, and interfering with the control of the apoptosis mechanism (5).

Thus, the main objective of our study evaluated the effects of the sucralose on the human lymphocytes cells and their subpopulations related to the immunologic system, evaluation aspects of cytotoxicity, genotoxicity, and mutagenic effects.

MATERIAL AND METHODS

Chemicals

We purchased Sterile Histopaque 1.077, RPMI 1640 modified with 20 mM HEPES and L-glutamine, inactivated Fetal Bovine Serum (FBS), Phytohemagglutinin-M (PHA-M), penicillin/streptomycin, gentamicin solution, and Sucralose (CAS n.56038-13-2) from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were analytical grade and stored according to the manufacturer's instructions.

Preparation of human lymphocyte culture

We prepare the lymphocytes cultures using 10mL of venous blood taken from the median cubital vein of a volunteer donor who had not consumed alcohol, smoked, or taken any medication that could interfere with the scientific results in the last 72 h, according to the Organization for Economic Cooperation and Development (6) (Federal University of Pampa Ethics Committee, 27045614.0.0000.5323). Lymphocytes were isolated with Histopaque1077® (Sigma-Aldrich, St. Louis, EUA) and transferred to the culture medium containing 9mL of RPMI 1640 supplemented with 20% fetal bovine serum and 1% streptomycin/penicillin, as described in previous work (7, 8). The cells were conditioned in culture flasks and placed in at 37ºC in a 5% CO_2 environment for up to 48 hours.

Selection of concentrations for tests

Due to a lack of studies on the compound, we chose the doses to allow abroadspectrum evaluation, which enabled the determination of a median lethal concentration (LC_{50}) (8) Therefore, we initially tested concentrations of 1 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL e 1000 μg/mL in cultures of lymphocytes, and, after analysis of cell proliferation and cell viability, through loss of membrane integrity using the Trypan Blue method (7).

Treatment of cultures.

All cultures received sucralose diluted in RPMI 1640 in the final volume of 1000μL. The groups tested were the following: Negative Control (NC) with phosphate buffer pH 7.4, Positive Control (PC) with Colchicine 10 μM, and three concentrations of the sucralose. These concentrations were chosen, as mentioned, based on the LC_{50}. We performed all tests in triplicate and analyzed all samples at 24 hours and 48 hours after exposure to sucralose.

Alkaline comet assay

We performed this test using the technique described by Singh (9) and Rice-Evans (10). We present the result as a DNA damage index (ID), based on the migration pattern of the nucleoid fragments. We calculated the DNA damage from cells with different damage classifications; the damage index ranges from 0 (100 cells x 0 when no damage occurred) to 400 (100 cells x 4, when maximum damage occurred).

Chromosomal aberration test and mitotic index

We conduct the chromosomal aberration assay as described by Yunis (11) with modifications. In a few words, 10^6 PBMC/ flask was stimulated with 1% (v/v) of PHA-M and incubated at 37ºC for 48 h. After this period, we exposed the lymphocytes to different concentrations of sucralose for three hours. KayoMAX Colcemid solution was added to cultures for three hours before the harvesting period for cell cycle blockade. Then, we centrifuged the cultures at 1000 x g for 5 min, the cell pellet was resuspended in KCl 0.075 M and centrifuged at 1000 x f for 5 min. Following PBMC were fixed with cold methanol: acetic acid (3:1) on pre-chilled microscopic slides. Subsequently, we stained the slides with 5%
Giemsa and scored 300 well-spread metaphases for the presence of structural and numeric chromosomal alterations. Additionally, we calculated the mitotic index for each treatment as the number os dividing cell per 100 cells.

Determination of lymphocyte subpopulations

Lymphocytes were suspended in the medium at a density of 0.5 x 10^6 / per 12-well plate well and exposed to sucralose, in three different concentrations determined by the doseeffect curve for 24 h. We assessed the specific toxicity for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocyte subpopulations by the Countess II FL Automatic Cell Counter (Thermo Fisher, Massachusetts, USA) equipped with EVOS ™ Light Cube GFP fluorescence cube and addition of Anti-CD3 (FICT), Anti-CD4 (FITC) and Anti-CD8 (FITC) previously in the samples according to the manufacturer's specifications (Abcam, Cambridge, MA).

In Silico analyses

In a complementary way and to search for possible methods of action in humans, the compound Sucralose was submitted to a series of computational tests (In Silico) through the platforms: ProTox (12), Way2Drug (13), and GeneCards (14). The addresses of these platforms are in the references.

Statistical analysis

We expressed the data as mean ± standard deviation and performed all analyses using specific statistical software. For the cytotoxicity curve, we used non-linear regression, and for the other analyzes, we used a one-way analysis of variance (ANOVA), followed by Tukey's Post-Hoc test. Results with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

We tested a wide range of concentrations from 1 µg/mL to 500 µg/mL to obtain the LC_{50}. Using the nonlinear regression calculation (we do not show the curves here), we achieved the LC_{50} for sucralose of 78.51 µg/mL. From this result, we determined the concentrations that would be tested in the following experiments for analysis of cytotoxic, mutagenic and genotoxic, being relevant to obtain concentrations that allow cell viability always higher than 80% (10). Thus, we defined the test concentrations of the experiments as 1 µg/mL, 10 µg/mL, and 50 µg/mL.

Figure 02 represents cell proliferation (A) and cell viability (B). Sucralose had a decrease in the number of lymphocytes in the concentration of 50 µg/mL in the order of approximately 48%. Regarding viability, even at the highest concentration tested, we did not observe any effects on viability.

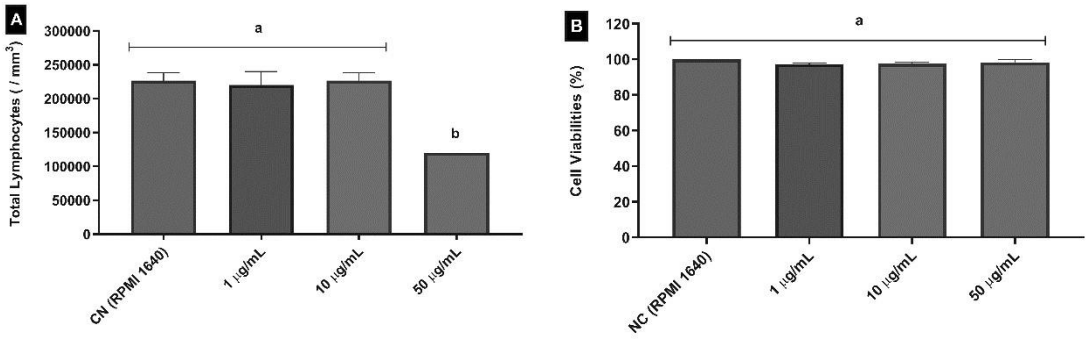

Figure 02: Evaluation of cell proliferation (A) and cell viability (B) of human lymphocytes exposed to Sucralose in cell culture. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In Figure 03, we present the effects of sucralose in lymphocyte subpopulations $CD3^+$, $CD4^+$, $CD8^+$, and double population ($CD4^+CD8^+$), respectively.

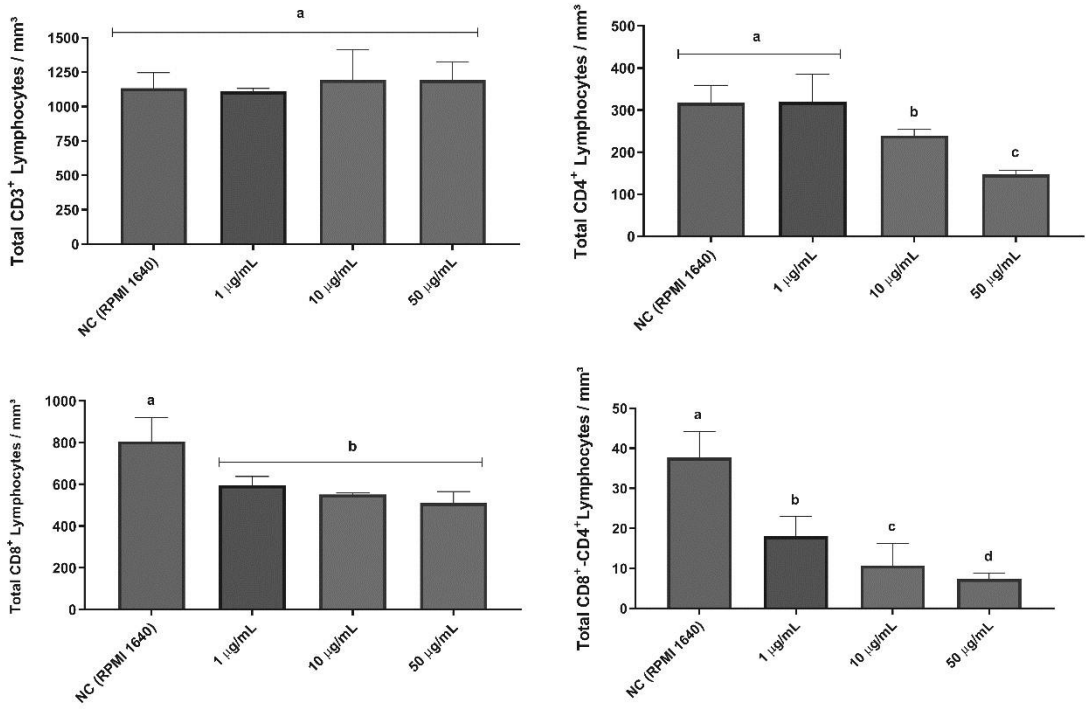

Figure 03: Effect of sucralose in lymphocytes subpopulations $CD3^+$(A), $CD4^+$(B), $CD8^+$(C), and Double population ($CD4^+CD8^+$) (D). We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

We observed that Sucralose did not show inhibition of lymphocytes T $CD3^+$ in any of the tested concentrations. Regarding the amounts of lymphocytes T $CD4^+$, sucralose caused a decrease in concentrations of 10 µg/mL (↓~25,12%) and 50 µg/mL (↓~54,62%). In the subpopulation of lymphocytes of T $CD8^+$, the sweetener Sucralose showed cell depletion at all concentrations tested, without statistical difference between the concentrations, around 31.4%. In double population lymphocytes, we found that Sucralose, decrease this subpopulation in all tested concentrations, ranging from 51.35% to 80.54% from the lowest to the highest concentration.

A study of Bessler and Djaldetti (15) matches our data. In this work, commercial sucralose was used, in the concentration of 10 µg/mL in the culture of PBMC and colon cancer cells, by test XTT not affect cell viability. According to the work of Van Eyk (16) found that the sucralose sweetener in the concentration of 10mM (equivalent to 4000µg/mL for Sucralose), in the cells Caco-2 and HT-29 obtained a decrease in cell viability by the MTT and did not experience cell decrease in HEK - 293.

Figure 08 shows the DNA damage indexes caused by sucralose. Sucralose showed average damage of approximately 62% higher than the negative control group in concentrations of 10 µg/mL and 50 µg/mL without statistical difference between concentrations.

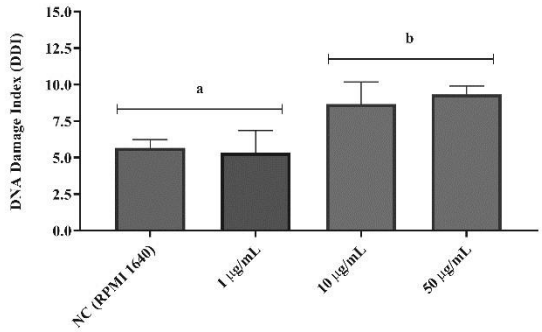

Figure 04: Effect of Sucralose on DNA damage of cultured PBMC. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In turn, Table 01 and figure 05 shows the data from the Chromosomal Instability test. In this test, we counted 300 cells in metaphase and evaluated numerical and structural chromosomal changes such as karyopicnose, chamfering, breaking, ligament, and others. Sucralose in all concentrations (1, 10 and 50 µg/mL) caused structural changes proportionally to the concentration. The type of structural change observed in all cases was the karyopicnose.

Table 01: Numerical and structural chromosomal aberrations induced by sucralose in cultured human lymphocytes.

Group	Mitotic metaphases index	Totals counted	Normal metaphases	Numerical changes	Structural changes	Types of changes founded
NC	1.40 ± 0.2[a]	300	299 (Figure 05A)	01	00	----------
1 µg/mL	1.30 ± 0.1[a]	300	269	00	31	Karyopicnose (Figure 05B)
10 µg/mL	1.30 ± 0.1[a]	300	236	00	64	Karyopicnose (Figure 05C)
50 µg/mL	1.30 ± 0.1[a]	300	188	00	112	Karyopicnose

Legend: We express the data as mean ± standard deviation, performed in triplicates.

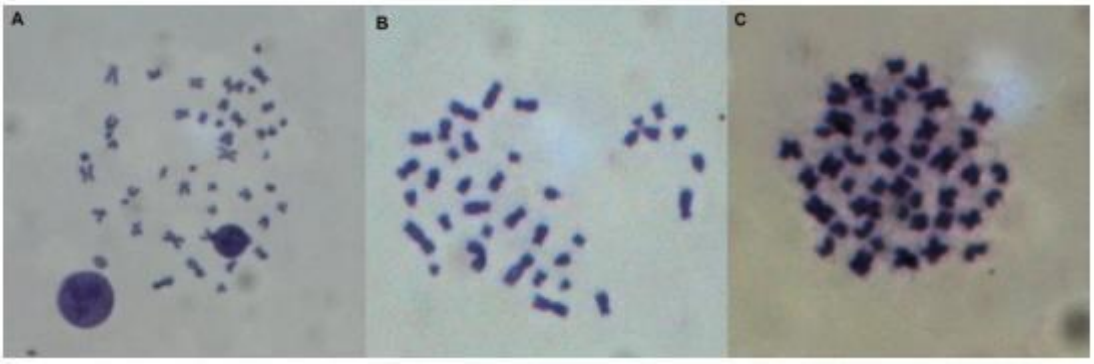

Figure 05: Chromosomal aberrations induced by Sucralose in cultured human lymphocytes. We took the photos under an optical microscope at 400x magnification. From A to C, examples of regular chromosome sets (A) and structural changes (B and C).

In a test, Shastry and Yatheesh (17), using concentrations of acceptable daily sucralose intake in rats for three weeks, showed that in the Ames test, there was no mutagenic effect.

In Silico methods analyzed complementarily, interactions with genes related to the cell cycle or with genetic aspects of the cell. We compiled the results in Table 02.

Table 02: Prediction of the effects of sucralose on genes involved with survival and cell genetics.

Gene	Resulted in effect	%*	The biological effect of the protein encoded by the gene
MAPK8	↓	90,9	Involved in cell proliferation and differentiation.
APTX	↓	89,6	Related to DNA repair.
EID1	↑	83.9	Involved with Histone regulation.

Legend: ↓ = reduces expression of the protein encoded by the gene; ↑ = increased expression of the protein encoded by the gene; * percentage chance of the compound causing expected interference.

Although we do not have a reported mechanism in the literature of why these compounds cause these effects shown here, we described a possible alternative by the evaluation In Silico. According to these tests, the primary mechanism involved with DNA

damage or changes is related to the modulation of gene expression, interfering with proteins related to replication, maintenance, and repair of DNA damage.

This article presents the results of an evidence-based approach to human health safety in the use of sucralose sweetener. Finally, this study points out that sucralose has cytotoxic, genotoxic, and mutagenic effects in the concentrations and conditions tested in human lymphocyte cell culture.

ACKNOWLEDGMENTS

Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) supported this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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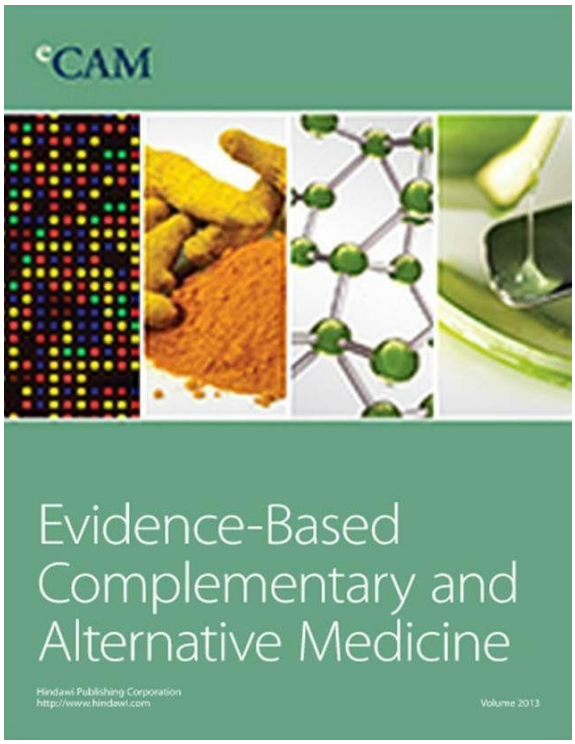
ANEXOS I (Cont...)

MANUSCRITO(S) SUBMETIDOS OU EM FASE DE REDAÇÃO REFERENTES A ESTA DISSERTAÇÃO

MANUSCRITO II

STEVIOL, THE ACTIVE PRINCIPLE OF THE STEVIA SWEETENER, CAUSES REDUCTION OF THE CELLS OF THE IMMUNOLOGICAL SYSTEM EVEN CONSUMED IN LOW CONCENTRATIONS.

SUBMETIDO PARA



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STEVIOL, THE ACTIVE PRINCIPLE OF THE STEVIA SWEETENER, CAUSES REDUCTION OF THE CELLS OF THE IMMUNOLOGICAL SYSTEM EVEN CONSUMED IN LOW CONCENTRATIONS.

Thaís Pasqualli [1,2], Pamella Eduardha Espindola Chaves[1], Lavínia da Veiga Pereira[1], Élvio Adílio Serpa[1,2], Luís Flávio Souza de Oliveira [1,2], Michel Mansur Machado [1,2,*]

[1] TOXCEL - Grupo de Pesquisa em Toxicologia Celular, Universidade Federal do Pampa, Uruguaiana, Brasil;

[2] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Pampa, Uruguaiana, Brasil.

E-mail address:

Thaís Pasqualli thais.pasqualli@yahoo.com.br

Pamella Eduardha Espindola Chaves pamella.eduardha.chaves@gmail.com

Lavínia da Veiga Pereira lavinia-veiga@hotmail.com

Élvio Adílio Serpa serpaelvio@gmail.com

Luís Flávio Souza de Oliveira luisoliveira@unipampa.edu.br

Michel Mansur Machado michelmachado@unipampa.edu.br

* Send correspondence to: Prof. Dr. Michel Mansur Machado, Universidade Federal do Pampa – Campus Uruguaiana, BR 472, Km 585, Uruguaiana, RS, Brasil, CEP: 97.500-970.

Tel / Fax: 55 - 55 39110200.

E-mail address: michelmachado@unipampa.edu.br

ABSTRACT

Steviol is a natural diterpenoid glycoside isolated from *Stevia rebaudiana* Bertoni leaves and widely used as a non-caloric sweetener. In addition to their sweet taste, Steviol glycosides may also have some therapeutic benefits. There are few reports on the cytotoxicity of Steviol in human cells. Our objective was to test this sweetener under and at average concentrations of consumption, evaluating parameters of cytotoxicity, genotoxicity, and immunotoxicity. For this purpose, we made use of lymphocyte cultures and the analysis of their $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations. In a complementary way, the mechanism of action is proposed here by computational methods. Our results showed that the sweetener Steviol reduces the number of lymphocytes due to falls in the levels of the $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subpopulations. Besides, we observed an increase in the level of DNA damage and a gradual incidence of structural changes in the lymphocyte chromosomal sets. It was possible to propose that Steviol modulates gene expression, mainly interfering with the SESN1, NAP1L1, SOX4 and TREX1 genes. Although Steviol is used globally as a sweetener, its use should be cautious, as our study points out that Steviol has cytotoxic, genotoxic and mutagenic effects in the concentrations and conditions tested in the culture of human lymphocyte cells.

Keywords: Steviol; Stevia; *In vitro*; *In silico*; lymphocytes

1. INTRODUCTION

Because *Stevia rebaudiana* Bertoni is not caloric, it has benefits for human health and therapeutic properties such as antihyperglycemic, antihypertensive, antioxidant, anti-tumor, anti-diarrheal, diuretic, gastroprotective, and immunomodulatory (Prata et al. 2017).

Belonging to the Asteraceae family, *Stevia rebaudiana* has a high content of glycosides such as Rebaudioside A (Figure 1A), plus a flavor 100 to 300 times sweeter than sucrose, and it has extensively used in many types of foods, medications, drinks, cosmetics, in the production of wine and household chemicals. Steviol (Figure 1C) is a natural diterpenoid glycoside isolated from *Stevia rebaudiana* leaves, and the primary active metabolite, since Rebaudiosides and Stevioside (Figure 1B) are converted to Steviol after oral ingestion for their action in the body (Anker, Rafiq, and Jeppesen 2019).

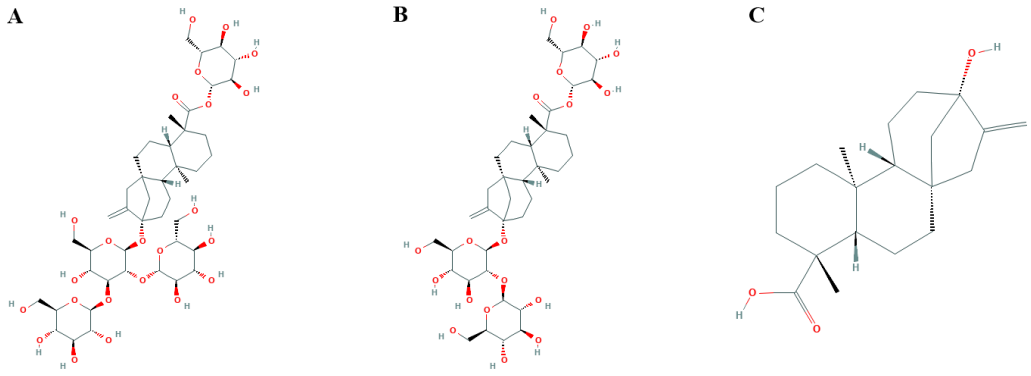

Figure 01: Main active principles of Stevia rebaudiana Bertoni. In (A) Rebaudioside A, in (B) Stevioside and in (C) Steviol.

In addition to their sweet taste, Steviol can also have a therapeutic benefit, as they prove to be an anti-inflammatory, anti-hyperglycemic, anti-hypertensive, anti-tumor, antidiarrheal, diuretic, and immunomodulatory activity (Panagiotou et al. 2018).

To date, few reports have studied the cytotoxicity of Steviol in human cells. According to Chen et al. (2018), there is a report that in U2OS cells (Osteosarcoma lineage), Steviol has anticancer activity.

Thus, the main objective of our study evaluated the effects of the Steviol on the human lymphocytes cells and their subpopulations related to the immunologic system, evaluation aspects of cytotoxicity, genotoxicity, and mutagenic effects.

2. MATERIAL AND METHODS

2.1 Chemicals:

We purchased Sterile Histopaque 1.077®, RPMI 1640 modified with 20 mM HEPES and Lglutamine, inactivated Fetal Bovine Serum (FBS), Phytohemagglutinin-M (PHA-M), penicillin/streptomycin, gentamicin solution, and Steviol sweetener (CAS n. 471-80-7) from the company Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents used were analytical grade and stored according to the manufacturer's instructions.

2.2 Preparation of human lymphocyte culture

We prepare the lymphocyte cultures using 10mL of venous blood taken from the median cubital vein, applied by a volunteer from a healthy, voluntary doctor who did not consume alcohol, smoked or took any medication that would interfere with medical results in the last
72 h, according to Organization for Economic Cooperation and Development (OECD 2014) (Federal University of Pampa Ethics Committee, 27045614.0.0000.5323). Then the blood was transferred to falcon tubes containing Histopaque-1077® (Sigma-Aldrich, St. Louis, USA) and transferred to the culture medium containing 9 mL of RPMI 1640 supplemented with 20% fetal

bovine serum and 1 % streptomycin/penicillin, as described in a previous study (Güez et al. 2017, Burow et al. 1998). All cells were conditioned in culture flasks and placed at 37ºC in a 5% CO_2 environment for up to 48 hours.

2.3 Selection of Concentrations for Tests

Due to the lack of studies on the compound, we have chosen the doses to allow broadspectrum evaluation, which allowed the determination of a median lethal concentration (LC_{50}). The doses used covered a wide spectrum, seeking to evaluate the largest possible range of use and were exposed to concentrations of 1 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL and 1000 μg/mL. The parameters analyzed were the membrane, using the Trypan Blue method (6). After these tests, we calculated the Lethal Concentration 50 % (LC_{50}), concentration where 50% of the lymphocytes were dead, using as a reference to calculate the doses for the other tests (de Moura Leão et al. 2018).

2.4 Treatment of cultures

All cultures received Steviol diluted in RPMI 1640 in the final volume of 1000μL. The groups tested were the following: Negative Control (NC) with phosphate buffer pH 7.4, Positive Control (PC) with Colchicine 10 μM, and three concentrations of the Steviol. These concentrations were chosen, as mentioned, based on the LC_{50}. We performed all tests in triplicate and analyzed all samples at 24 hours and 48 hours after exposure to Steviol.

2.5 Alkaline comet assay

We performed this test using the technique described by Singh et al. (1988) and Rice-Evans, Miller, and Paganga (1996). We present the result as a DNA damage index (ID), based on the migration pattern of the nucleoid fragments. We calculated the DNA damage from cells with

different damage classifications; the damage index ranges from 0 (100 cells x 0 when no damage occurred) to 400 (100 cells x 4, when maximum damage occurred).

2.6 Chromosomal aberration test and mitotic index

We conduct the chromosomal aberration assay as described by Yunis (1976) with modifications. In a few words, 1 X 10^6 lymphocytes/ flask was stimulated with 1% (v/v) of PHA-M and incubated at 37ºC for 48 h. After this period, we exposed the lymphocytes to different concentrations of Steviol for three hours. The KayoMAX® Colcemid solution was added to the cultures for three hours before the harvest period to block the cell cycle. Then, we centrifuged the cultures at 1000 x g for 5 min, the cell pellet was resuspended in KCl 0.075 M and centrifuged at 1000 x f for 5 min. Following lymphocytes were fixed with cold methanol: acetic acid (3:1) on pre-chilled microscopic slides. Subsequently, we stained the slides with 5% Giemsa and scored 300 well-spread metaphases for the presence of structural and numeric chromosomal alterations. Additionally, we calculated the mitotic index for each treatment as the number of dividing cells per 100 cells.

2.7 Determination of lymphocyte subpopulations

Lymphocytes were suspended in the medium at a density of 0.5 x 10^6 / per 12-well plate well and exposed to Steviol, in three different concentrations determined by the dose-effect curve for 24 h. We assessed the specific toxicity for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocyte subpopulations by the Countess II FL Automatic Cell Counter (Thermo Fisher, Massachusetts, USA) equipped with EVOS ™ Light Cube GFP fluorescence cube and addition of Anti-CD3 (FICT), Anti-CD4 (FITC) and Anti-CD8 (FITC) previously in the samples according to the manufacturer's specifications (Abcam, Cambridge, MA).

2.8 *In Silico* analyses

In a complementary way and to search for possible methods of action in humans, the compound Steviol was submitted to a series of computational tests (*In Silico*) through the platforms: ProTox (Drwal et al. 2014), Way2Drug (Filimonov et al. 2014), and GeneCards (Safran et al. 2010). The addresses of these platforms are in the references.

2.9 Statistical analysis

We expressed the data as mean ± standard deviation and performed all analyses using specific statistical software. For the cytotoxicity curve, we used non-linear regression, and for the other analyzes, we used a one-way analysis of variance (ANOVA), followed by Tukey's PostHoc test. Results with $p < 0.05$ were considered significant.

3. RESULTS AND DISCUSSION

We tested a wide range of concentrations from 1 µg/mL to 500 µg/mL to obtain the LC_{50}. Using the nonlinear regression calculation (we do not show the curves here), the LC_{50} obtained for Steviol was 178.7 µg/mL. From this result, we determined the concentrations for the next experiments (cytotoxic, mutagenic and genotoxic parameters), and that is relevant to obtain concentrations that allow cell viability always higher than 80%. Thus, we defined the test concentrations of the experiments as 1 µg/mL, 10 µg/mL and 50 µg/mL.

Figure 02 represents cell proliferation (A) and cell viability (B). Note that Steviol

had a decrease in the number of lymphocytes at a concentration of 50 µg/mL in the order of approximately 48%. Regarding viability, even at the highest concentration tested, we did not observe any effects on viability.

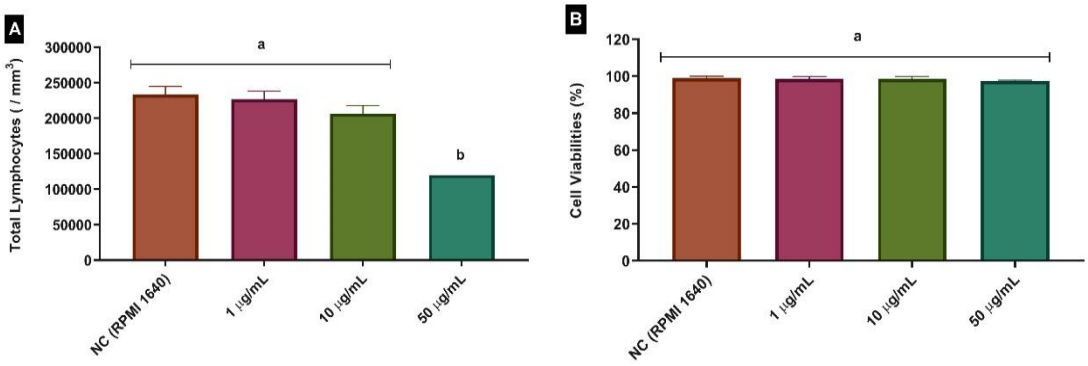

Figure 02: Evaluation of cell proliferation (A) and cell viability (B) of human lymphocytes exposed to Steviol in cell culture. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In Figure 03, we present the effects of Steviol in lymphocyte subpopulations $CD3^+$, $CD4^+$, $CD8^+$, and double population ($CD4^+CD8^+$), respectively.

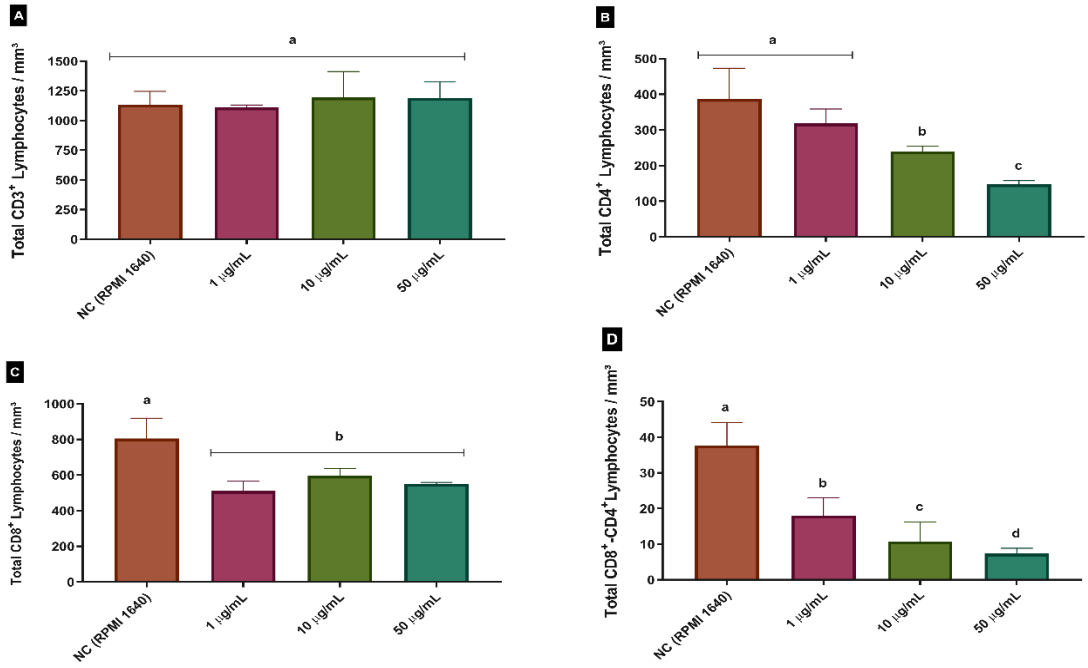

Figure 03: Effect of Steviol in lymphocytes subpopulations $CD3^+$(A), $CD4^+$(B), $CD8^+$(C), and Double population ($CD4^+CD8^+$) (D). We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$.

Different letters mean statistically different values. NC = Negative Control.

We observed that Steviol did not show inhibition of lymphocytes T $CD3^+$ in any of the tested concentrations (Figure 3A). Regarding the amounts of lymphocytes T $CD4^+$, Steviol (Figure 3B) caused a decrease in concentrations of 10 µg/mL (↓~35%) and 50 µg/mL (↓70%). In the $CD8^+$ T lymphocyte subpopulation, Steviol showed cell depletion of around 32%, but with no significant difference between the tested concentrations (Figure 3C). Double Population lymphocytes ($CD4^+CD8^+$) are young, immature cells that have not yet finished their cell maturation process (Zuckermann and Gaskins 1996). We identified that Steviol (Figure 3D) cause a cellular decrease, in all tested concentrations, for this subgroup of cells, ranging from 52.21% to 80.54% of depletions in the higher concentration.

In a study by Bessler and Djaldetti (2019) using commercial Stevia, at a concentration of 10 µg/mL in PBMC culture and colon cancer cells, by the XTT assay, the authors did not find effects on cell viability. As for the Steviol result, a finding demonstrates that in six different types of gastrointestinal cell cultures, it shows cytotoxicity by the MTT assay, however, in a concentration of 100 to 200 µg/mL (4).

Figure 04 shows the DNA damage indexes caused by the effects of sweeteners. The sweetener Steviol showed average damage of approximately 62% higher than the negative control group in the concentrations of 10 µg/mL and 50 µg/mL, with no statistical difference between the concentrations.

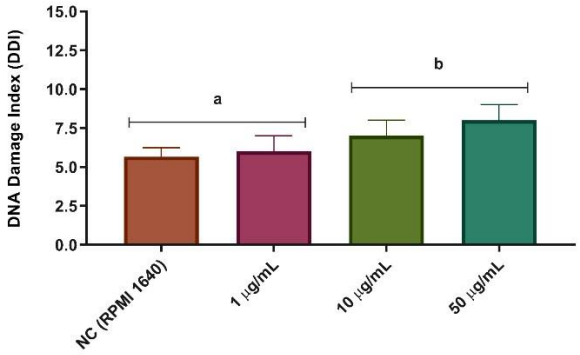

Figure 04: Effect of Steviol on DNA damage of cultured lymphocytes. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

Table 01 and Figure 5 show the data from the Chromosomal Instability test. In this test, we counted 300 cells in metaphase. We evaluated numerical and structural chromosomal changes, such as ringing, karyopyknosis, chamfering, breaking, ligament etc. With the

sweetener Steviol, we observed changes in the highest concentrations, 10 and 50 µg/mL. The type of structural alteration observed was karypycnose.

Table 01: Numerical and structural chromosomal aberrations induced by Steviol in cultured human lymphocytes.

Group	Mitotic Index	Totals Metaphases Counted	Normal Metaphases	Numerical Changes	Structural Changes	Type Of Change Found
CN	1, 40 ± 0.2a	300	299 (Figure 5A)	01	0 0	---------- ----
1 µg/mL	1, 30 ± 0,1a	300	300	00	0 0	Karypycnose (Figure 5B)
10 µg/mL	1, 20 ± 0,1a	300	251	00	4 9	Karypycnose (Figure 5B)
50 µg/mL	1, 10 ± 0,1a	300	213	00	8 7	Karypycnose

Legend: We express the data as mean ± standard deviation, performed in triplicates.

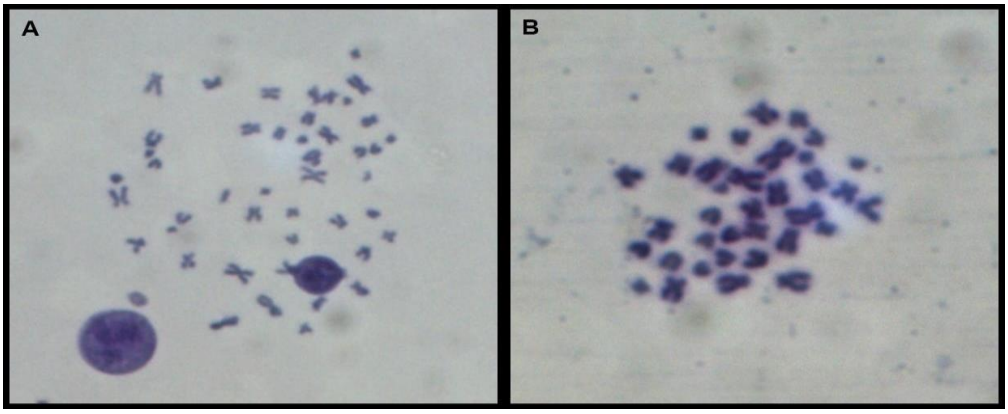

Figure 05: 400X optical microscope photo. From A to B, examples of regular chromosome sets (A) and structural changes - Karypycnose (B).

Other studies carried out the chromosomal aberration assay in human lymphocytes with Stevia and observed structural changes (breaks) in the concentrations from 1 to 16 µg/mL (Uçar et al. 2018).

Computational methods analyzed complementarily, interactions with genes related to the cell cycle, or with genetic aspects of the cell, and we present the results in Table 02.

Table 02: Prediction of the effects of Steviol on genes involved with survival and cell genetics.

Gene	Resulted in effect	%*	The biological effect of the protein encoded by the gene
SESN1	↓	93,2	Play a role in the cellular response to DNA damage.
NAP1L1	↓	87,5	It participates in DNA replication, chromatin modulation, and regulation of cell proliferation.
SOX4	↓	81,2	It is involved in the Apoptosis process.
TREX1	↓	78,9	Involved in the DNA repair process.

Legend: ↓ = reduces expression of the protein encoded by the gene; ↑ = increased expression of the protein encoded by the gene; * percentage chance of the compound causing expected interference.

Although we do not have a reported mechanism in the literature for these compounds to cause these effects shown here, a possible alternative is described by the *In-Silico* evaluation. According to these tests, as we can see in the table above, the primary mechanism involved with DNA damage or changes is related to the modulation of gene expression, interfering with proteins related to DNA replication, maintenance and repair.

Although Steviol is used globally as a sweetener in thousands of foods and beverages, its use should be cautious, as a study points out that Steviol has cytotoxic, genotoxic and

mutagenic effects in the concentrations and conditions tested in the culture of human lymphocyte cells.

ACKNOWLEDGMENTS

Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) supported this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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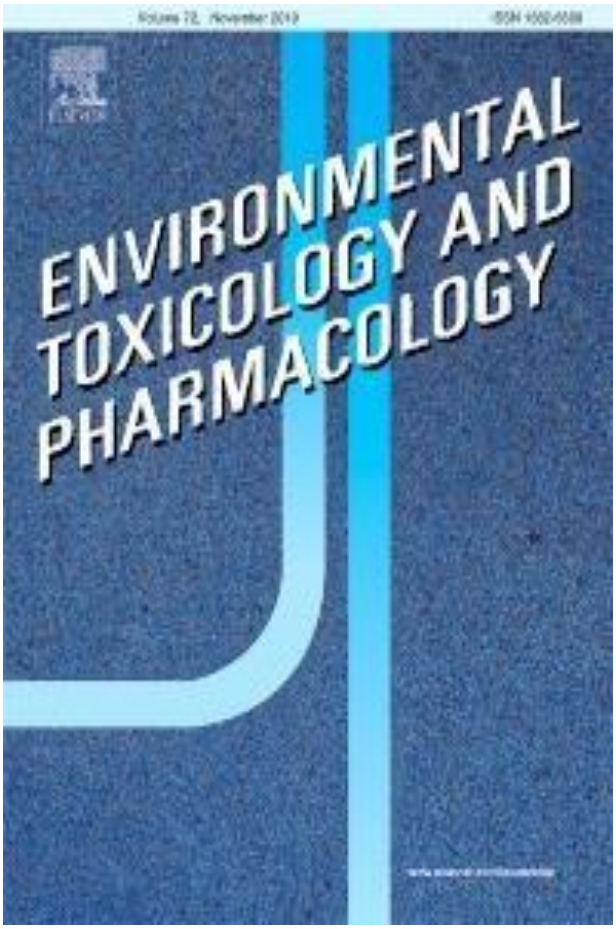
ANEXOS I (Cont...)

MANUSCRITO(S) SUBMETIDOS OU EM FASE DE REDAÇÃO REFERENTES A ESTA DISSERTAÇÃO

MANUSCRITO III

SACCHARIN IN THE DIET.
REDUCING CALORIES AND ALSO YOUR IMMUNE RESPONSE?

SUBMETIDO PARA



EM 04/05/2020

SACCHARIN IN THE DIET.

REDUCING CALORIES AND ALSO YOUR IMMUNE RESPONSE?

Short Title: EFFECTS OF SACCHARIN IN IMMUNE RESPONSE.

Thaís Pasqualli [1,2], Pamella Eduardha Espindola Chaves[1], Lavínia da Veiga Pereira[1], Élvio Adílio Serpa[1,2], Luís Flávio Souza de Oliveira [1,2], Michel Mansur Machado [1,2,*]

[1] TOXCEL - Grupo de Pesquisa em Toxicologia Celular, Universidade Federal do Pampa, Uruguaiana, Brasil;

[2] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Pampa, Uruguaiana, Brasil.

* Send correspondence to: Prof. Dr. Michel Mansur Machado, Universidade Federal do Pampa – Campus Uruguaiana, BR 472, Km 585, Uruguaiana, RS, Brasil, CEP: 97.500-970. Tel / Fax: 55 - 55 39110200. E-mail address: michelmachado@unipampa.edu.br; ORCID 0000-0002-7583-9332.

ABSTRACT

Accidentally discovered in 1879, Saccharin is the oldest artificial sweetener in use. Our goal was to test this sweetener at average consumption concentrations, evaluating parameters of cytotoxicity, genotoxicity, and immunotoxicity. For this, we used lymphocyte cultures and the analysis of their $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations. In a complementary way, the mechanism of action is proposed here by computational methods. We observed an increase in the level of DNA damage and an incidence of structural changes in the lymphocyte chromosomal sets. Also, our results showed that saccharin reduces total lymphocytes due to falls in levels of the $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subpopulations. It was possible to propose that saccharin modulates gene expression, interfering, mainly the SIVA1 and TFAM genes. Finally, this study addresses results that demonstrate the use of sweeteners with the safety of human health.

Keywords: Saccharin; gene regulation; lymphocytes; CD8; CD4.

1. INTRODUCTION

Search for low energy foods and the demand for sweeteners, like saccharin, is increasing. This fact may be related to health care because of the diseases that high sucrose consumption causes, such as obesity, diabetes, and dental caries (1). Accidentally discovered in 1879 by Ira Remsen and Constantine Fahlberg, Saccharin (Figure 1) is the oldest artificial sweetener in use today (2).

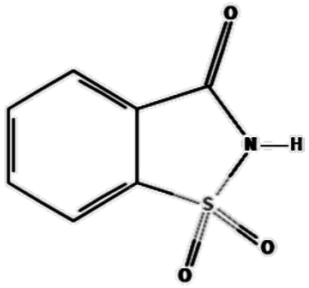


Figure 1: Chemical structure of Saccharin.

Saccharin has a sweetening power 200 to 700 times higher than sucrose, high stability, it is not hygroscopic and has zero caloric power. Also, it is not metabolized in the human digestive system and is rapidly excreted in the urine, not accumulating in the human body (2). However, it has a bitter and metallic taste, which makes it often used in combination with other sweeteners (3).

Considering the importance attached to the use of sweeteners, the present work proposes to analyze the cytotoxic effects, genotoxic and mutagenic effects of saccharin, using human lymphocytes cultured in the laboratory.

2. MATERIAL AND METHODS

2.1 Chemicals

We purchased Sterile Histopaque 1.077, RPMI 1640 modified with 20 mM HEPES and L-glutamine, inactivated Fetal Bovine Serum (FBS), Phytohemagglutinin-M (PHA-M), penicillin/streptomycin, gentamicin solution, and Saccharin (CAS n. 81-07-2) from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were analytical grade and stored according to the manufacturer's instructions.

2.2 Preparation of human lymphocyte culture

We prepare the lymphocytes cultures using 10mL of venous blood taken from the median cubital vein of a volunteer donor who had not consumed alcohol, smoked, or taken any medication that could interfere with the scientific results in the last 72h, according to the Organization for Economic Cooperation and Development (4) (Federal University of Pampa Ethics Committee, 27045614.0.0000.5323). Lymphocytes were isolated with Histopaque-1077® (Sigma-Aldrich, St. Louis, EUA) and transferred to the culture medium containing 9mL of RPMI 1640 supplemented with 20% fetal bovine serum and 1% streptomycin/penicillin, as described in previous work (5, 6). The cells were conditioned in culture flasks and placed in at 37ºC in a 5% CO_2 environment for up to 48 hours.

2.3 Selection of concentrations for tests

Due to a lack of studies on the compound, we chose the doses to allow abroad-spectrum evaluation, which enabled the determination of a median lethal concentration (LC_{50}) (7). Therefore, we initially tested concentrations of 1 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL e

1000 μg/mL in cultures of lymphocytes, and, after analysis of cell proliferation and cell viability, through the loss of membrane integrity using the Trypan Blue method (6).

2.4 Treatment of test cultures.

The test cultures received saccharin diluted in RPMI 1640 in the final volume of 1000μL. The groups tested were the following: Negative Control (NC) with phosphate buffer pH 7.4, Positive Control (PC) with Colchicine 10 μM, and three concentrations of the sucralose. These concentrations were chosen, as mentioned, based on decimal fractions of the LC_{50}. We performed all tests in triplicate and analyzed all samples at 24 hours and 48 hours after exposure to saccharin.

2.5 Alkaline comet assay

We performed this test using the technique described by Singh, McCoy (7) and Rice-Evans, Miller (8). We present the result as a DNA damage index (ID), based on the migration pattern of the nucleoid fragments. We calculated the DNA damage from cells with different damage classifications; the damage index ranges from 0 (100 cells x 0 when no damage occurred) to 400 (100 cells x 4, when maximum damage occurred).

2.6 Chromosomal aberration test and mitotic index

We conduct the chromosomal aberration assay as described by Yunis (9) with modifications. In a few words, 10^6 PBMC/ flask was stimulated with 1% (v/v) of PHA-M and incubated at 37ºC for 48h. After this period, we exposed the lymphocytes to different concentrations of saccharin for three hours. KayoMAX Colcemid solution was added to cultures for three hours before the harvesting period for cell cycle blockade. Then, we centrifuged the cultures at 1000 x g for 5 min, the cell pellet was resuspended in KCl 0.075 M and centrifuged at 1000 x f for 5 min. Following PBMC were fixed with

cold methanol: acetic acid (3:1) on pre-chilled microscopic slides. Subsequently, we stained the slides with 5% Giemsa and scored 300 well-spread metaphases for the presence of structural and numeric chromosomal alterations. Additionally, we calculated the mitotic index for each treatment as the number of dividing cell per 100 cells.

2.7 Determination of lymphocyte subpopulations

Lymphocytes were suspended in the medium at a density of 0.5 x 10^6 / per 12-well plate well and exposed to saccharin, in three different concentrations determined by the dose-effect curve for 24 h. We assessed the specific toxicity for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocyte subpopulations by the Countess II FL Automatic Cell Counter (Thermo Fisher, Massachusetts, USA) equipped with EVOS ™ Light Cube GFP fluorescence cube and addition of Anti-CD3 (FICT), Anti-CD4 (FITC) and Anti-CD8 (FITC) previously in the samples according to the manufacturer's specifications (Abcam, Cambridge, MA).

***2.8 In Silico* analyses**

In a complementary way and to search for possible methods of action in humans, the compound saccharin was submitted to a series of computational tests (*In Silico*) through the platforms: ProTox (10), Way2Drug (11), and GeneCards (12). The addresses of these platforms are in the references.

2.9 Statistical analysis

We expressed the data as mean ± standard deviation and performed all analyses using specific statistical software. For the cytotoxicity curve, we used non-linear regression, and for the other

analyzes, we used a one-way analysis of variance (ANOVA), followed by Tukey's Post-Hoc test. Results with $p < 0.05$ were considered significant.

3. RESULTS AND DISCUSSION

We tested a wide range of concentrations from 1 μg/mL to 500 μg/mL to obtain the LC_{50}. Using the nonlinear regression calculation (*we do not show the curves here*), we achieved the LC_{50} for saccharin of 321.2 μg/mL. From this result, we determined the concentrations that would be tested in the following experiments for analysis of cytotoxic, mutagenic and genotoxic, being relevant to obtain concentrations that allow cell viability always higher than 80% (8). Thus, we defined the test concentrations of the experiments as 1 μg/mL, 10 μg/mL, and 50 μg/mL.

Figure 02 represents cell proliferation (A) and cell viability (B), note that saccharin did not affect proliferation nor cell viability at the concentrations tested compared to the negative control.

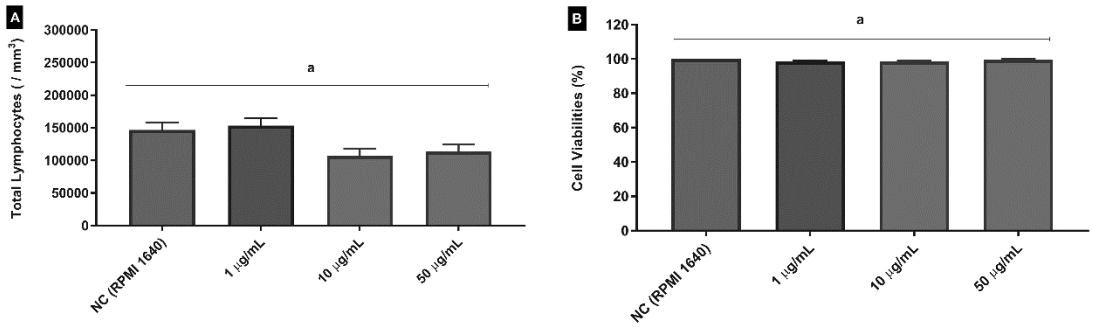


Figure 02: Evaluation of cell proliferation (A) and cell viability (B) of human lymphocytes exposed to Saccharin in cell culture. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In Figure 03, we present the effects of saccharin in lymphocyte subpopulations $CD3^+$, $CD4^+$, $CD8^+$, and double population ($CD4^+CD8^+$), respectively.

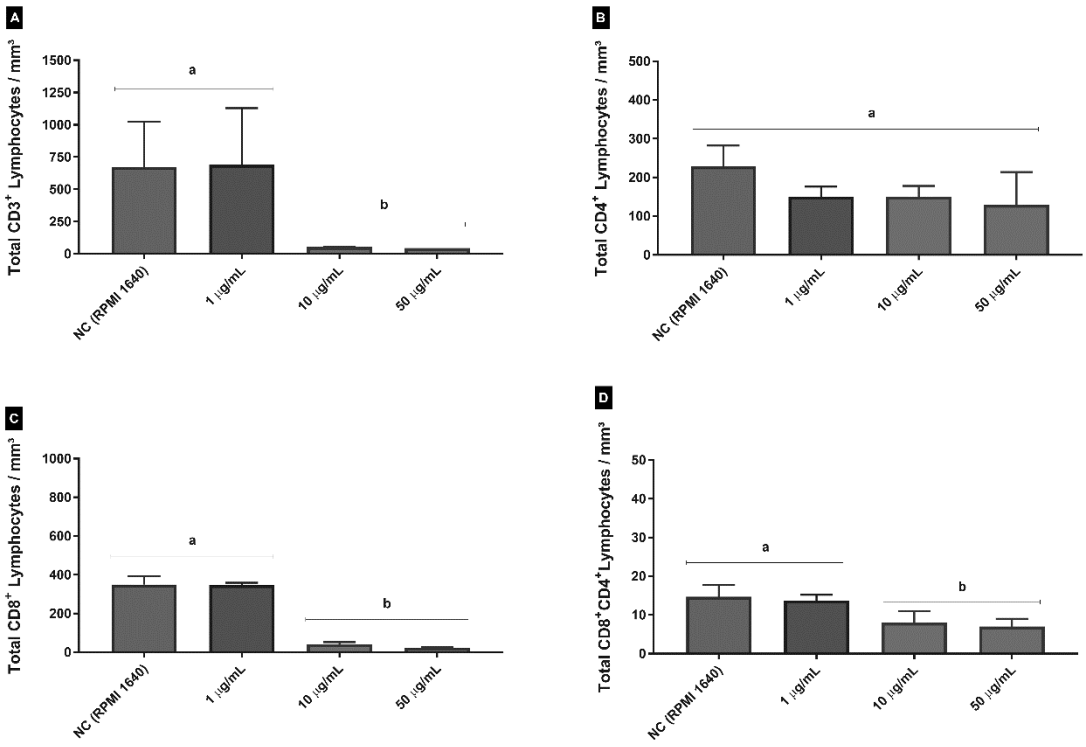


Figure 03: Effect of saccharin in lymphocytes subpopulations CD3+(A), CD4+(B), CD8+(C), and Double population (CD4+CD8+) (D). We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

Saccharin caused an inhibition of Lymphocytes T $CD3^+$ at concentrations 10 µg/mL and 50 µg/mL. These decreases were significant and reached values around 91% and 93%, respectively. Regarding the amounts of lymphocytes T $CD4^+$, Saccharin did not cause significant

changes in any of the tested concentrations. Also, saccharin caused a high decrease in lymphocytes $CD8^+$ subset, at concentrations 10 μg / mL and 50 μg / mL, ranging from 32% to 94%. In double population lymphocytes, saccharin also caused cell inhibition only at concentrations of 10 μg / mL and 50 μg / mL.

The study of Bessler, Djaldetti (13) has divergent results from ours. They test Saccharin at a concentration of 10 μg/mL in the culture of PBMC and colon cancer cells by the XTT assay showing no effect on cell viability.

According to Van Eyk (14), saccharin at a concentration of 10mM in Caco-2 and HT-29 cells caused a decrease in cell viability by MTT assay but not a decrease in HEK-293.

Figure 4 shows the increases in the DNA damage rates caused by the effects of saccharin. Saccharin increases damage in all tested concentrations, ranging from an increase of 2.5X in lower concentration to a higher 13x the highest concentration.

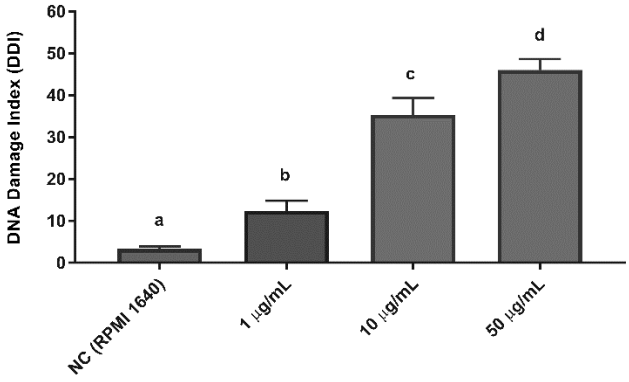


Figure 04: Effect of Saccharin on DNA damage of cultured PBMC. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

Other researchers reinforce our results. Van Eyk (14) found that saccharin showed DNA damage by the alkaline comet assay in a colon cell culture (Caco-2 e HT-29) and kidney of (HEK-293) in concentrations of 0.1mM, 1.0mM, and 10mM.

In turn, Table 01 and figure 05 shows the data from the Chromosomal Instability test. In this test, we counted 300 cells in metaphase and evaluated numerical and structural chromosomal changes such as karyopicnose, chamfering, breaking, ligament, and others.

Saccharin showed changes in the highest concentrations, 10 and 50 μg / mL. The kind of abnormality observed was karyopyknosis and centromeric fusions. However, you do not observe any numerical changes.

Table 01: Total of numerical and structural chromosomal aberrations induced by Steviol in cultured human lymphocytes.

Group	Mitotic Index	Totals Metaphases Counted	Normal Metaphases	Numerical Changes	Structural Changes	Type Of Change Found
CN	1.40 ± 0.2[a]	300	299 (Figure 05A)	01	00	----------
1 μg/mL	0.80 ± 0.1[a]	300	300	00	00	----------
10 μg/mL	0.7 ± 0.2[b]	300	241	00	59	*Centromeric Fusion* (Figure 05B)
50 μg/mL	0.5 ± 0.1[c]	300	298	00	102	Karypycnose (Figure 05C)

Legend: We express the data as mean ± standard deviation, performed in triplicates.

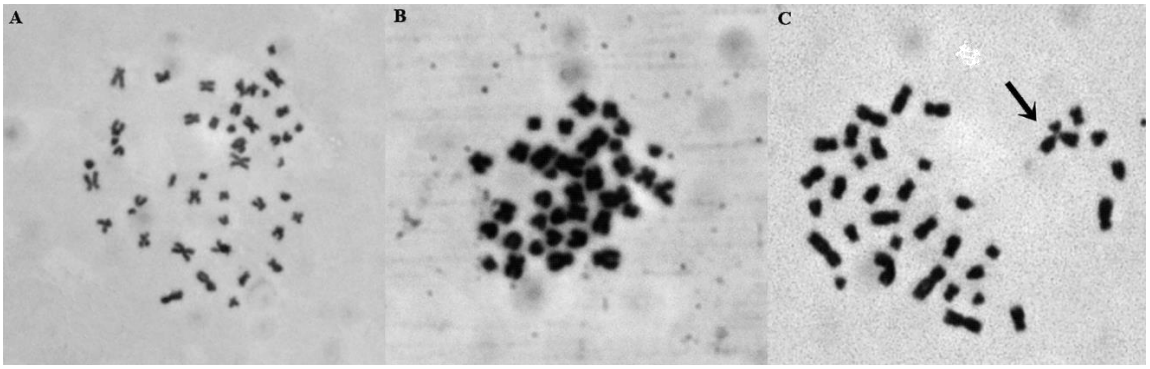


Figure 05: Chromosomal aberrations induced by Saccharin in cultured human lymphocytes. We took the photos under an optical microscope at 400x magnification. From A to C, examples of regular chromosome sets (A) and structural changes (B and C).

A mutagenicity study using the Ames test (15) demonstrated that Saccharin was negative at concentrations of 50, 100, and 200 μg / plate.

We used *In Silico* Methods to analyze possible interactions with genes related to the cell cycle or with genetic aspects of the cell. We compiled the results in Table 02.

Table 02: Prediction of the effects of Steviol on genes involved with survival and cell genetics.

Gene	Resulted in effect	%*	The biological effect of the protein encoded by the gene
SIVA1	↓	96.1	Gene involved in cell cycle regulation.
TFAM	↓	77.8	Involved in the DNA repair process.

Legend: ↓ = reduces expression of the protein encoded by the gene; * percentage chance of the compound causing expected interference.

Although we do not have a reported mechanism in the literature for these compounds to cause these effects shown here, a possible alternative is described by the *In-Silico* evaluation. According to these tests, as we can see in the table above, the primary mechanism involved with DNA damage or changes related to modulation of gene expression, interfering with proteins involved in replication, maintenance, and repair of DNA.

4. CONCLUSION

This study serves as a basis for relating the use of saccharin with human health safety at low concentrations. We concluded that Saccharin has cytotoxic, genotoxic, and mutagenic effects in the concentrations and conditions tested in human lymphocyte cell culture.

ACKNOWLEDGMENTS

National Council for Scientific and Technological Development and Coordination for the Improvement of Higher Education Personnel (CAPES) supported this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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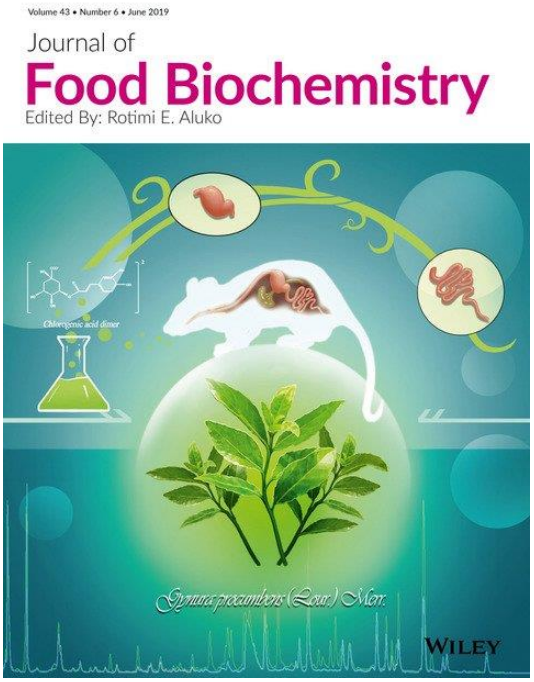
ANEXOS I (Cont...)

MANUSCRITO(S) SUBMETIDOS OU EM FASE DE REDAÇÃO REFERENTES A ESTA DISSERTAÇÃO

MANUSCRITO IV

THE USE OF FRUCTOSE AS A SWEETENER.
IS IT REALLY A SAFE ALTERNATIVE FOR OUR IMMUNE SYSTEM?

SUBMETIDO PARA



EM 29/05/2020

THE USE OF FRUCTOSE AS A SWEETENER.
IS IT A SAFE ALTERNATIVE FOR OUR IMMUNE SYSTEM?

Running Title: FRUCTOSE AND IMMUNE SYSTEM.

Thaís Pasqualli [1,2]; Pamella Eduardha Espindola Chaves[1]; Lavínia da Veiga Pereira[1]; Élvio Adílio Serpa[1,2]; Luís Flávio Souza de Oliveira [1,2]; Michel Mansur Machado [1,2,*]

[1] TOXCEL - Grupo de Pesquisa em Toxicologia Celular, Universidade Federal do Pampa, Uruguaiana, Brasil;

[2] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Pampa, Uruguaiana, Brasil.

* Send correspondence to: Prof. Dr. Michel Mansur Machado, Universidade Federal do Pampa – Campus Uruguaiana, BR 472, Km 585, Uruguaiana, RS, Brasil, CEP: 97.500-970. Tel / Fax: 55 - 55 39110200; E-mail address: michelmachado@unipampa.edu.br; ORCID 0000-0002-7583-9332.

ABSTRACT

Fructose is a constituent of sucrose and other polymers referred to as inulin or fructans. We can find Fructose in cereals, vegetables, and honey. It has the property of being 1.5 times sweeter than sucrose. Our objective was to test this sweetener under and at average concentrations of consumption, evaluating parameters of cytotoxicity, genotoxicity, and immunotoxicity. For this purpose, we made use of lymphocyte cultures and the analysis of their $CD4^+$ and $CD8^+$ subpopulations. In a complementary way, the mechanism of action is proposed here by computational methods. Our results showed that Fructose reduces lymphocytes due to the levels of the $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subpopulations. Besides, we observed an increase in DNA damage. It was possible to propose that Fructose modulates gene expression, mainly interfering with the MAPK8, APTX, TUBGCP3, and LST1 genes. Although Fructose is used globally as a sweetener, its use should be cautious, as our study points out that Steviol has cytotoxic, genotoxic and mutagenic effects in the concentrations and conditions tested in the culture of human lymphocyte cells.

Keywords: Fructose; *In vitro*; *In silico*; lymphocytes; CD4; CD8.

1. INTRODUCTION

Fructose (Figure 1) is a constituent of sucrose (β-D-fructofuranosyl α-D-glucopyranoside) and other polymers referred to as inulin or fructans. We can find Fructose in cereals, vegetables, and honey. It has the property of being 1.5 times sweeter than sucrose (1). Excessive use of Fructose as a sweetener by the food industry made to reach an increase in the numbers of cases of metabolic syndrome, obesity, hypertension, *Diabetes mellitus*, insulin resistance, and also cases of hepatic steatosis (2).

Fructose exerts personal effects on lipid and carbohydrate metabolism. For its potential bioenergetic not compensated, the Fructose can be more harmful to health. In addition to that, several studies in humans support the link between high intake of Fructose and hyperglycemia, insulin resistance, and type 2 diabetes (3).

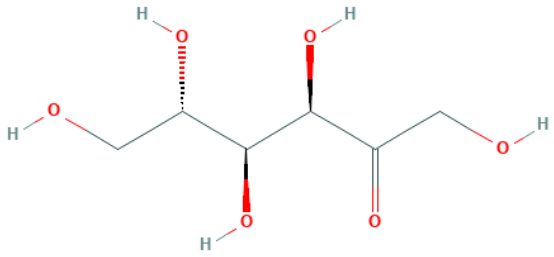


Figure 1 - The chemical structure of Fructose.

Fructose is absorbed in the intestine and metabolized by the human liver, via GLUT5 transporters, it can produce glucose, glycogen, lactate, and pyruvate, which makes it an essential substrate for metabolic interconversion (4). Fructose intake was also associated with higher blood pressure levels in adolescents and adults without a previous history of hypertension. Another study showed that the consumption of fructose results in decreased production of the hormones insulin and leptin, that are

involved in the regulation of energy homeostasis and body adiposity. In such a way, that in the long run, the consumption of diets rich in and Fructose can lead to increased energy consumption, weight gain, and obesity (5)**.**

Thus, the main objective of our study evaluated the effects of the Fructose on the human lymphocytes cells and their subpopulations related to the immunologic system, evaluation aspects of cytotoxicity, genotoxicity, and mutagenic effects.

2. MATERIAL AND METHODS

2.1 Chemicals: We purchased Sterile Histopaque 1.077®, RPMI 1640 modified with 20 mM HEPES and L-glutamine, inactivated Fetal Bovine Serum (FBS), Phytohemagglutinin-M (PHA-M), penicillin/streptomycin, gentamicin solution, and Fructose sweetener (CAS n. 57-48-7) from the company Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents used were analytical grade and stored according to the manufacturer's instructions.

2.2 Preparation of human lymphocyte culture: We prepare the lymphocytes cultures using 10mL of venous blood taken from the median cubital vein of a volunteer donor who had not consumed alcohol, smoked, or taken any medication that could interfere with the scientific results in the last 72 h, according to the Organization for Economic Cooperation and Development (6) (Federal University of Pampa Ethics Committee, 27045614.0.0000.5323). Lymphocytes were isolated with Histopaque-1077® (Sigma-Aldrich, St. Louis, EUA) and transferred to the culture medium containing 9mL of RPMI 1640 supplemented with 20% fetal bovine serum and 1%

streptomycin/penicillin, as described in previous work (7, 8). The cells were conditioned in culture flasks and placed in at 37ºC in a 5% CO_2 environment for up to 48 hours.

2.3 Selection of concentrations for tests: Due to a lack of studies on the compound, we chose the doses to allow abroad-spectrum evaluation, which enabled the determination of a median lethal concentration (LC_{50}) (8) Therefore, we initially tested concentrations of 1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL e 1000 µg/mL in cultures of lymphocytes, and, after analysis of cell proliferation and cell viability, through the loss of membrane integrity using the Trypan Blue method (9).

2.4 Treatment of cultures: All cultures received sucralose diluted in RPMI 1640 in the final volume of 1000μL. The groups tested were the following: Negative Control (NC) with phosphate buffer pH 7.4, Positive Control (PC) with Colchicine 10 μM, and three concentrations of the Fructose. These concentrations were chosen, as mentioned, based on the LC_{50}. We performed all tests in triplicate and analyzed all samples at 24 hours and 48 hours after exposure to sucralose.

2.5 Alkaline comet assay: We performed this test using the technique described by Singh, McCoy (10), and Rice-Evans, Miller (11). We present the result as a DNA damage index (ID), based on the migration pattern of the nucleoid fragments. We calculated the DNA damage from cells with different damage classifications; the damage index ranges from 0 (100 cells x 0 when no damage occurred) to 400 (100 cells x 4, when maximum damage occurred).

2.6 Chromosomal aberration test and mitotic index: We conduct the chromosomal aberration assay as described by Yunis (12) with modifications. In a few words, 1 X 10^6 lymphocytes/ flask was stimulated with 1% (v/v) of PHA-M and incubated at 37ºC for 48 h. After this period, we exposed the lymphocytes to different concentrations of Fructose for three hours. The KayoMAX® Colcemid solution was added to the cultures for three hours before the harvest period to block the cell cycle. Then, we centrifuged the cultures at 1000 x g for 5 min, the cell pellet was resuspended in KCl 0.075 M and centrifuged at 1000 x f for 5 min. We fixed the lymphocytes with cold methanol: acetic acid (3:1) on pre-chilled microscopic slides. Subsequently, we stained the slides with 5% Giemsa and scored 300 well-spread metaphases for the presence of structural and numeric chromosomal alterations. Additionally, we calculated the mitotic index for each treatment as the number of dividing cells per 100 cells.

2.7 Determination of lymphocyte subpopulations: Lymphocytes were suspended in the medium at a density of 0.5 x 10^6 / per 12-well plate well and exposed to Fructose, in three different concentrations determined by the dose-effect curve for 24 h. We assessed the specific toxicity for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocyte subpopulations by the Countess II FL Automatic Cell Counter (13) (Thermo Fisher, Massachusetts, USA) equipped with EVOS ™ Light Cube GFP fluorescence cube and addition of Anti-CD3 (FICT), Anti-CD4 (FITC) and Anti-CD8 (FITC) previously in the samples according to the manufacturer's specifications (Abcam, Cambridge, MA).

2.8 *In Silico* analyses: The canonical form of Fructose was searched in the PubChem database (14) and submitted to a series of computational tests (*In Silico*)

through the platforms: ProTox (15), Way2Drug (16), and GeneCards (17). These platforms jointly evaluate the possible interactions between the Sucralose molecule and 14,700 human genes. Those actions (upregulations and downregulation above 80%) and related to genes involved with the parameters analyzed here were summarized.

2.9 Statistical analysis: We expressed the data as mean ± standard deviation and performed all analyses using specific statistical software. For the cytotoxicity curve, we used nonlinear regression, and for the other analyzes, we used a one-way analysis of variance (ANOVA), followed by Tukey's Post-Hoc test. Results with $p < 0.05$ were considered significant.

3. RESULTS AND DISCUSSION

We tested a wide range of concentrations from 1 μg/mL to 500 μg/mL to obtain the LC_{50}. Using the nonlinear regression calculation (*we do not show the curves here*), we achieved the LC_{50} for Fructose higher than 1,000 μg/mL. From this result, we determined the concentrations that would be tested in the following experiments for analysis of cytotoxic, mutagenic and genotoxic, being relevant to obtain concentrations that allow cell viability always higher than 80% (6). Thus, we choose the test concentrations of the experiments as 1 μg/mL, 10 μg/mL, and 50 μg/mL, more like food concentrations.

Figure 2 represents cell proliferation (A) and cell viability (B). We note that Fructose does not affect the proliferation and even cell viability in the tested concentrations compare with the negative control.

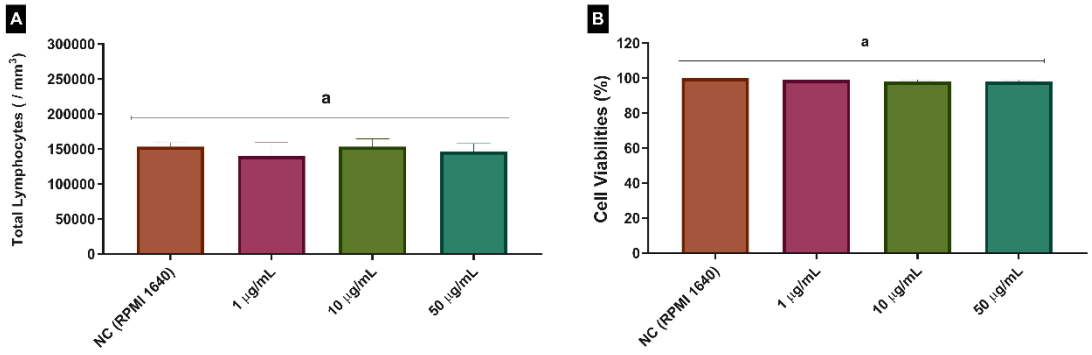


Figure 2: Evaluation of cell proliferation (A) and cell viability (B) of human lymphocytes exposed to Fructose in cell culture. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In Figure 3, we present the effects of Fructose in lymphocyte subpopulations $CD3^+$, $CD4^+$, $CD8^+$, and double population ($CD4^+CD8^+$).

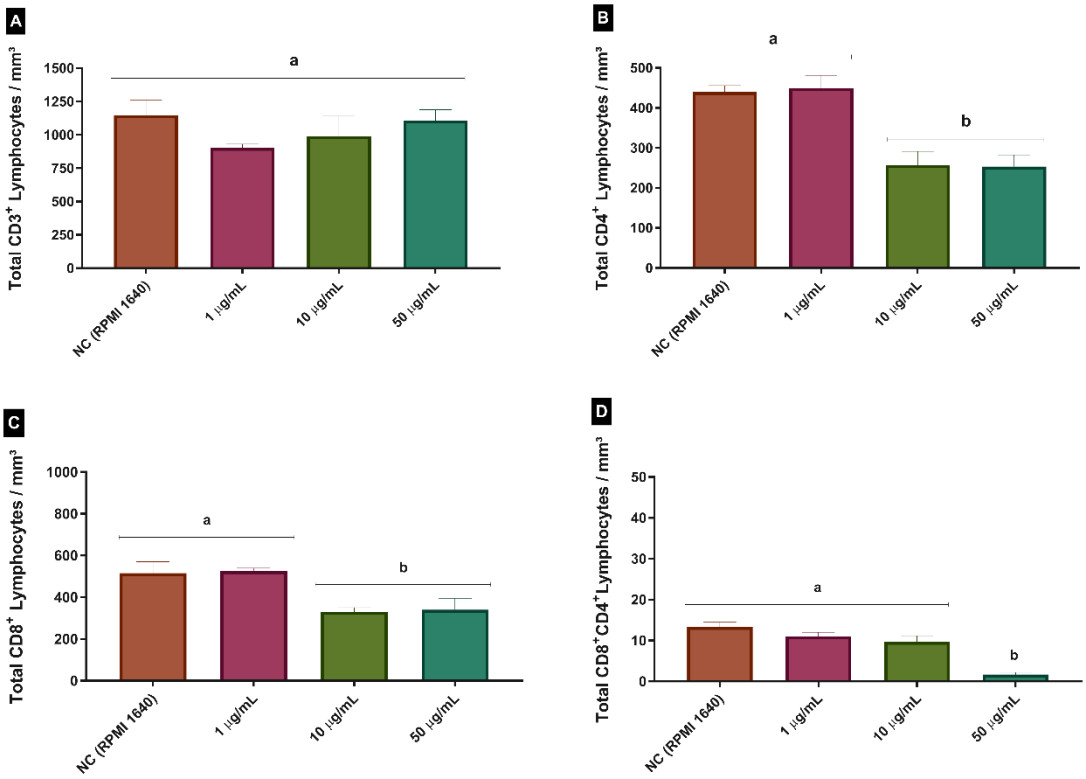


Figure 3: Effect of fructose in lymphocytes subpopulations $CD3^+$(A), $CD4^+$(B), $CD8^+$(C), and Double population ($CD4^+CD8^+$) (D). We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

Fructose showed no inhibition of Lymphocytes T $CD3^+$ in any of the tested concentrations. Lymphocytes T $CD4^+$ and $CD8^+$ showed a decrease in the concentrations of 10 µg/mL and 50 µg/mL, with reductions of around 42% for CD4 and 35% for CD8 (with no difference between concentrations 10 and 50 µg / mL in the two tests). Lymphocytes double-population also decreases, but this only occurs at a concentration of 50 µg/mL (with a drastic reduction of around 87.5%).

In Figure 4 are illustrate the DNA damage rates caused by the effects of Fructose. It showed average damage of approximately 62% higher than the negative control group at concentrations 10 µg/mL and 50 µg/mL.

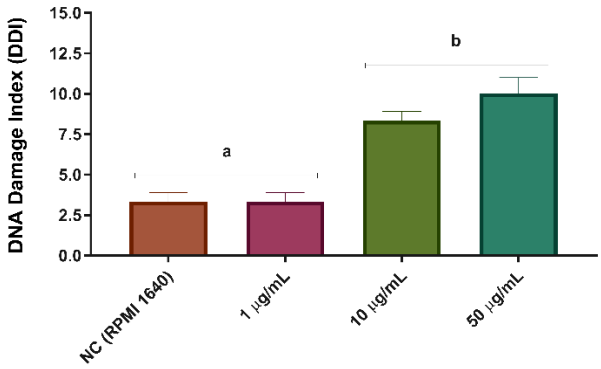


Figure 4: Effect of Fructose on DNA damage of cultured Lymphocytes. We expressed the data as mean ± standard deviation, n=3, performed in triplicates, and analyzed by ANOVA followed by Tukey's post hoc. We considered significant results with $p<0.05$. Different letters mean statistically different values. NC = Negative Control.

In turn, Table 1 shows the data from the Chromosomal Instability test. In this test, we counted 300 cells in metaphase and evaluated numerical and structural chromosomal changes such as karyopicnose, chamfering, breaking, ligament, and others. None of the concentrations from Fructose made alterations in analyzed parameters.

Table 1: Numerical and structural chromosomal aberrations induced by Fructose in cultured human lymphocytes.

Group	Mitotic Index	Totals Metaphases Counted	Normal Metaphases	Numerical Changes	Structural Changes
CN	1.40 ± 0.2^{a}	300	299^{a}	01	00
1 µg/mL	0.80 ± 0.1^{a}	300	300^{a}	00	00
10 µg/mL	0.7 ± 0.2^{b}	300	299^{a}	01	00
50 µg/mL	0.5 ± 0.1^{c}	300	299^{a}	01	00

Legend: We express the data as mean ± standard deviation, performed in triplicates.

A study has shown that an overload of sucrose (concentration of 6.9%, 13.8%, or 34.8% (w / w) in the diet of rats, showed a mutation in the colon cells in the highest concentrations, but not in the liver cells (18).

In Silico methods analyzed complementarily, showed interactions with genes related to the cell cycle or with genetic aspects of the cell. We compiled the results in Table 02.

Table 2: Prediction of the effects of Fructose on genes involved with survival and cell genetics.

Gene	Resulted in effect	%*	The biological effect of the protein encoded by the gene
MAPK8	↓	91.6	Involved in cell proliferation and differentiation.
APTX	↓	90.5	Related to DNA repair.
TUBGCP3	↓	82.9	Related to cell cycle control.
LST1	↑	86.4	Inhibit lymphocytes proliferation

Legend: ↓ = reduces expression of the protein encoded by the gene; * percentage chance of the compound causing expected interference.

Although we do not have a reported mechanism in the literature for these compounds to cause the effects showed here, we described a possible alternative by the evaluation *In Silico*. According to these tests, as we can see in the table above, the primary mechanism involved with DNA damage or changes is related to the modulation of gene expression, interfering with proteins related to DNA replication, maintenance, and repair.

4. CONCLUSION

Our study serves as a basis to relate the use of Fructose safely human health. We can conclude that Saccharin has cytotoxic, genotoxic, and mutagenic effects in the concentrations and conditions tested in human lymphocyte cell culture.

ACKNOWLEDGMENTS

National Council for Scientific and Technological Development and Coordination for the Improvement of Higher Education Personnel (CAPES) supported this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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ANEXOS II

PUBLICAÇÕES REFERENTES A ESTA DISSERTAÇÃO DE MESTRADO

REPÚBLICA FEDERATIVA DO BRASIL

UNIJUÍ - UNIVERSIDADE REGIONAL DO NOROESTE DO ESTADO DO RIO GRANDE DO SUL

IJUÍ - SANTA ROSA - PANAMBI - TRÊS PASSOS

CERTIFICADO

Certificamos que o trabalho **Análise das Possíveis Atividades da Stevia Rebaudiana no Organismo Humano Utilizando Modelo Computacional (*In Silico*)** de autoria de **Lavinia da Veiga Pereira, Thais Pasqualli, Elvio Adílio Serpa, Emanoeli da Rosa, Luís Flávio Souza de Oliveira e Michel Mansur Machado** foi apresentado no **6º Congresso Internacional em Saúde - Vigilância em Saúde: Ações de Promoção, prevenção, diagnóstico e tratamento**, promovido pelo Departamento de Ciências da Vida - DCVida da Universidade Regional do Noroeste do Estado do Rio Grande do Sul - UNIJUÍ, no período de 14 a 17 de maio de 2019, em Ijuí, pelo que lhe é conferido o presente certificado.

Ijuí (RS), 5 de junho de 2019.

Cátia S. Gehlke de Medeiros
Secretária Acadêmica

Cristina Eliza Pozzobon
Vice-Reitora de Graduação

11º SIEPE

EDUCAÇÃO E CIÊNCIA PARA O DESENVOLVIMENTO SUSTENTÁVEL
22 a 24 de outubro de 2019
Fronteira da Paz

EDUCACIÓN Y CIENCIA PARA EL DESARROLLO SOSTENIBLE
22 al 24 de octubre de 2019
Frontera de la Paz

CERTIFICADO

Certificamos que o trabalho intitulado **AVALIAÇÃO DA GENOTOXICIDADE DO ADOÇANTE SUCRALOSE EM LINFÓCITOS HUMANOS CULTIVADOS**, de autoria de **Thais Pasqualli, Michel Mansur Machado, Anelise Santos Soares, Elvio Adílio Serpa, Pamella Eduardha Espindola Chaves, Luís Flávio Souza De Oliveira**, na categoria Pesquisa, na modalidade Apresentação Oral, foi apresentado na Mostra Científica do 11º Salão Internacional de Ensino, Pesquisa e Extensão - SIEPE, em Santana do Livramento/RS.

Santana do Livramento, 24 de outubro de 2019.

Marco Antonio Fontoura Hansen
Reitor da Universidade Federal do Pampa

A autenticidade deste documento pode ser verificada através da URL: https://sgce.unipampa.edu.br/certificados/validar/4E13F71A

XIV CONGRESSO DA ASSOCIAÇÃO BRASILEIRA DE MUTAGÊNESE E GENÔMICA AMBIENTAL

CERTIFICADO

Certificamos que o trabalho intitulado

CITOTOXIC EFFECT OF SACRARINE SWEETENER IN CULTURED HUMAN LYMPHOCYTES

de autoria de Thais Pasqualli; Jéssica Limberguer; Anelise Santos Soares; Pamella Eduardha Chaves; Lavinia Veiga; Elvio Adilio Serpa; Luis Flávio Souza de Oliveira; Luisa Zuravski; Michel Mansur Machado; foi apresentado durante o XIV Congresso da Associação Brasileira de Mutagênese e Genômica Ambiental, na forma de pôster.

Bento Gonçalves / RS, 06 de junho de 2019.

Juliana da Silva
Presidente MutaGen-Brasil

Mário Sérgio Mantovani
Secretário MutaGen-Brasil

APOIO

FAPERGS CAPES CNPq

III SIMPÓSIO INTEGRADO DOS PPGs UNIPAMPA/URUGUAIANA

16 - 18 de Outubro, 2019 - UNIPAMPA, RS

CERTIFICADO

Certificamos que **Thais Pasqualli** apresentou o trabalho intitulado **Citotoxicidade do Adoçante Sucralose em Linfócitos Humanos Cultivados – Da alimentação ao risco a Imunidade!** de autoria de Thais Pasqualli e Michel Mansur Machado na modalidade **pôster** no **III Simpósio Integrado dos PPGs UNIPAMPA/Campus Uruguaiana**, realizado entre os dias 16 e 18 de outubro de 2019 em Uruguaiana, RS.

Pâmela Mello Carpes
Coordenadora

CIÊNCIA ANIMAL UNIPAMPA EDUCAÇÃO EM CIÊNCIAS: QUÍMICA DA VIDA E SAÚDE PPG química

unipampa

II SIMPÓSIO INTEGRADO DOS PPGs
UNIPAMPA / URUGUAIANA
III SIMPÓSIO GAÚCHO DE INOVAÇÃO EM SAÚDE
8 à 10 de Outubro de 2018

unipampa
Universidade Federal do Pamp

- CERTIFICADO -

Certificamos que **Thais Pasqualli** apresentou o trabalho **Análise das possíveis atividades da Stevia Rebaudiana no organismo humano utilizando modelo computacional (in silico)** , de coautoria de **Michel Mansur Machado**, modalidade **Pôster** no **II Simpósio Integrado dos PPGs e III Simpósio Gaúcho de Inovação em Saúde da UNIPAMPA/Campus Uruguaiana**, realizado nos dias 09 e 10 de Outubro de 2018, na Universidade Federal do Pampa Campus Uruguaiana.

Profa. Dra. Sandra Elisa Haas
Coordenadora do Simpósio

Bagé, 28/05/2019.

CAPES UNIPAMPA CIÊNCIA ANIMAL PPG bioquímica EDUCAÇÃO EM CIÊNCIA QUÍMICA DA VIDA E SAÚ