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Evaluations of the possible mutagenic and genotoxic effects of 2-ACBs: by-products generated from irradiated foods

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Abstract: This study investigates the potential mutagenic and genotoxic effects of 2-Alkylcyclobutanones (2-ACBs), by-products formed in irradiated foods. 2-ACBs are compounds derived from the irradiation of fat-containing foods, with recognized genotoxic potential. The research focused on the compounds 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB), evaluating their mutagenicity through the micronucleus assay in hepatic cell lines (HepG2, BRL3A, and HTC) and genotoxicity through the Ames test using five bacterial strains (TA-98, TA-100, TA-1535, TA-1537, and *WP2uvrA*). Results from the Ames test indicated that 2-dDCB and 2-tDCB did not significantly increase mutagenic reversion rates, while the micronucleus assays showed no genotoxic damage in the tested cell lines. It is concluded that, at the evaluated concentrations, the compounds 2-dDCB and 2-tDCB do not exhibit mutagenic or genotoxic potential, supporting the safety of irradiated foods. However, further research is recommended to assess long-term effects and different irradiation conditions.

Keywords: Food Irradiation, Alkylcyclobutanones (2-ACBs), 2-dodecylcyclobutanone (2-dDCB), 2-tetradecylcyclobutanone (2-tDCB), Ames Test, Micronucleus Assay

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Avaliações dos possíveis efeitos mutagênicos e genotóxicos dos 2-ACBs: subprodutos gerados a partir de alimentos irradiados

Resumo: Este estudo investiga os possíveis efeitos mutagênicos e genotóxicos dos subprodutos 2-Alquilciclobutanonas (2-ACBs), formados em alimentos irradiados. As 2-ACBs são compostos derivados da irradiação de alimentos contendo gordura, com reconhecido potencial genotóxico. A pesquisa centrou-se nos compostos 2-dodecilciclobutanona (2-dDCB) e 2-tetradecilciclobutanona (2-tDCB), avaliando sua mutagenicidade por meio do ensaio de micronúcleo em linhagens celulares hepáticas (HepG2, BRL3A e HTC) e genotoxicidade através do ensaio de Ames utilizando cinco cepas bacterianas (TA-98, TA-100, TA-1535, TA-1537 e *WP2uvrA*). Os resultados do ensaio de Ames indicaram que 2-dDCB e 2-tDCB não aumentaram significativamente as taxas de reversão mutagênica, enquanto os ensaios de micronúcleo mostraram ausência de danos genotóxicos nas linhagens celulares testadas. Conclui-se que, nas concentrações avaliadas, os compostos 2-dDCB e 2-tDCB não apresentam potencial mutagênico ou genotóxico, corroborando a segurança dos alimentos irradiados. Contudo, recomenda-se a realização de pesquisas adicionais para avaliar os efeitos a longo prazo e sob diferentes condições de irradiação.

Palavras-chave: Alimento Irradiado, 2-Alcilciclobutanonas (2-ACBs), 2-Dodecilciclobutanonas (2-dDCBs), 2-Tetradecilciclobutanonas (2-tDCBs), Teste de Ames, Ensaio de Micronucleos

1. INTRODUCTION

Food irradiation is a well-established method for preserving food by effectively reducing microbial load and extending shelf life without producing chemical residues. This process employs gamma radiation, which disrupts the DNA of microorganisms, inhibiting their ability to reproduce and thereby enhancing food safety [1, 2]. However, concerns have emerged regarding the formation of 2-Alkylcyclobutanones (2-ACBs), unique radiolytic products generated in fat-containing foods during irradiation [3].

The potential genotoxicity and mutagenicity of 2-ACBs, specifically 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB), have been subjects of increasing scientific scrutiny. Initial studies assessed the cytotoxicity of these compounds in hepatic cells, indicating the need for further detailed investigations into their genetic impacts using robust assays such as the Ames test and micronucleus assay [4, 5].

The Ames test, a widely accepted method for evaluating mutagenicity, utilizes specific bacterial strains (TA-98, TA-100, TA-1535, TA-1537, and *WP2uvrA*) to detect mutations caused by chemical compounds. This test has proven effective in identifying mutagenic agents in various substances, including irradiated food components [6]. Delinceé and Pool-Zobel (1998) identified genotoxic properties in 2-ACBs formed during the irradiation of fatty foods, raising health concerns [5]. Additionally, Chinthalapally (2003) questioned whether irradiated foods could cause or promote colon cancer, further emphasizing the need for comprehensive evaluations [7].

Micronucleus assays in mammalian cells, such as HepG2, BRL3A, and HTC, provide critical insights into the genotoxic effects of 2-ACBs by detecting the formation of micronuclei, indicative of chromosomal damage [8, 9]. These assays are essential for evaluating the genotoxic potential of radiolytic products in a more biologically relevant context.

Studies have highlighted the importance of monitoring 2-ACBs as indicators of irradiation doses in foods, emphasizing the need for rigorous safety assessments. Gadgil et

al. (2002) demonstrated the presence of these compounds in irradiated beef burgers, reinforcing the need for analytical methods to accurately detect and quantify 2-ACBs [10].

Song et al. (2014) conducted a critical review on the toxicological safety of 2-ACBs, highlighting the complexities of their interactions with biological systems and the importance of comprehensive toxicological assessments [11]. This review pointed to gaps in the current understanding and called for further research to elucidate the mechanisms of 2-ACB-induced toxicity.

Mahadevan et al. (2011) explored the genetic toxicology of various food components, providing insights into the mechanisms through which 2-ACBs may exert their effects. Continued research in this area is crucial for understanding the full spectrum of health implications associated with consuming irradiated foods [12].

A study published in 2022 provided an updated overview of the detection of 2-alkylcyclobutanones (2-ACBs) in irradiated foods. The research emphasized advancements in analytical techniques and highlighted the necessity for standardized detection methods to ensure food safety. This comprehensive review underscored the critical role of improved methodologies in accurately identifying 2-ACBs, thereby enhancing the safety and regulatory compliance of irradiated foods [13].

This study aims to deepen the understanding of the mutagenic and genotoxic potential of 2-ACBs, focusing on their impact on genetic material in both bacterial and mammalian cells. By providing robust evidence and addressing existing knowledge gaps, this research seeks to inform regulatory standards and promote safe practices in food irradiation [14-16].

In conclusion, while food irradiation offers significant benefits for food preservation and safety, the formation of 2-ACBs presents a critical challenge that requires thorough scientific investigation. Ensuring the safety of irradiated foods necessitates ongoing research and rigorous evaluation of these compounds' genotoxic and mutagenic effects to protect public health.

2. MATERIALS AND METHODS

2.1. Ames Test

The chemical purity of 2-dDCB is 99.2% and of 2-tDCB is 99.1%, both synthesized by Fluka Analytical and purchased from Sigma-Aldrich (USA). The tested doses were based on the study by Yamakage et al. (2014), using lower concentrations to avoid precipitation, with DMSO as the solvent. A volume of 0.1 mL of the solution per plate was used. The concentrations tested were 0.83, 0.27, and 0.09 mg/plate for both compounds, with approximate dilutions in µM per plate being 3.5, 1.10, 0.37 for 2-dDCB and 3.10, 1.00, 0.33 for 2-tDCB. The exposure concentrations depend on the diffusion capacity within the plate. The tests were performed at the Laboratory of Genotoxicity in Microorganisms - LGM - Center for the Development of Nuclear Technology / CDTN - CNEN Belo Horizonte – MG [16].

Cell lines Used

Cell line used in this study were purchased from Molecular Toxicology Inc. Moltox®, Boone, North Carolina, 28607, USA.

Five different bacterial strains were used to assess genotoxicity for both 2-dDCB and 2-tDCB. The strains used were: TA-1535, TA-1537, TA-98 and TA-100 from *Salmonella typhimurium* to detect point mutations at the Guanine-Cytosine (G-C). *WP2-uvrA* strain of *Escherichia coli* was used to detect point mutations at the Adenine-Thymine (A-T) sites [17].

Inoculum Preparation

Stock cultures were thawed and resuspended in Oxoid Nutrient Broth 2. The inoculum was shaken at 37°C and 100 RPM. Growth was monitored by spectrophotometry at an OD of 0.660 nm, reaching 1 to 2 x 10^9 CFU/mL.

Negative Control

Following OECD and FDA recommendations, DMSO was used as the negative control [18].

Positive Control

Positive controls were based on publications by OECD, FDA, CETESB, and Mortelmans and Zeiger (2000). Different reagents were used depending on the presence or absence of metabolic activation (S9 Mix), with quantities applied per plate as shown in Tables 1 and 2 [18, 19].

S9 Fraction and S9-mix Solution Preparation

The mouse liver homogenate (S9 fraction) was purchased from Molotolec Molecular Toxicology, Inc. The S9-mix solution (10%) was prepared and kept on ice on the day of the experiment.

Nutrient Broth and Minimal Agar Plates

Nutrient broth was prepared with Oxoid Nutrient Broth No. 2 and distilled water. Minimal agar was prepared with Vogel-Bonner E solution and glucose agar. Top agar was prepared with Bacto-agar, NaCl, distilled water, and supplemented with histidine and biotin for *S. typhimurium*, and tryptophan for *E. coli*. After solidification, plates were incubated at 37°C for 48 hours. At the end of the term, the number of revertant colonies on each plate was counted. The number of colonies observed on the plates containing the 2-dDCB and 2-tDCB was compared with the positive and negative controls.

Table 1 - Positive controls used in all bacterial strains studied, including concentration per plate in the tests with no metabolic activation

Strain	With absence of metabolic activation	
	Substance Control (+)	µg/plate
TA-98	2-Nitrofluorene	10
TA-100	Sodium Azide	5
TA-1535	Sodium Azide	5
TA-1537	ICR-191	1
*WP2-uvr*A	4-nitroquinoline-N-oxide	5

Table 2 - Positive controls used in all bacterial strains studied, including plate concentration in the assays in the presence of metabolic activation

Strain	With activation of metabolic presence	
	Substance Control (+)	µg/plate
TA-98	2-Aminoanthracene	5
TA-100	2-Aminoanthracene	5
TA-1535	2-Aminoanthracene	10
TA-1537	2-Aminoanthracene	10
*WP2-uvr*A	2-Aminoanthracene	15

Statistical analysis

Data analyses were performed using the Dunnett-t-test, according to recommendations of the U.K. Environmental Mutagen Society [20] is the best method to statistically evaluate the reverse mutation test in bacteria.

2.2. Micronucleus Assay

In the micronucleus (MN) assay, Bemis et al. (2016) [21] and Bryce et al. (2010) [22] protocol was used with some modifications.

Solubilization of test compounds

2-ACBs were solubilized in ethanol at a final concentration of 2%. The stock solution was prepared, at a concentration of 500 µM, from this one, the other two concentrations were prepared, 100 and 300 µM.

Cell lines Used

Cell lines used in the MN assay (BRL 3A, HepG2, HTC) were from the ICB/USP Department of Cell Biology and Development of the Institute of Biomedical Sciences - Universidade de São Paulo,

These cells were kept in culture bottles in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, packed in the oven under 5% CO2 atmosphere at 37 ° C.

After reaching near confluence (approximately 80 to 90% of the cell population), the cells were peaked, and sub cultured, and only after the third passage that they were used in the experiments.

The assay

After counting, the cells were resuspended in culture medium and distributed into 48 wells of a 96-well plate, with each well containing 100 µL of $5x10^3$ BRL 3A cells, $8x10^3$ HepG2 cells, and $3x10^3$ HTC cells. All assays were performed in quadruplicate, and the plates were incubated for 48 hours.

Pre-prepared solutions of test substances (2-ACBs), positive, negative, and blank controls were added after the cell growth period. The culture medium was withdrawn, and 100 µL of each solution was added to their respective wells (100, 300, and 500 µM of the test compounds), including positive, negative, and blank controls, until all 48 wells were filled. Colchicine (Sigma Aldrich CAS 64-86-8) was diluted in culture medium to a final concentration of 1.1 µg/mL and used as the positive control. The negative control was a 0.9% NaCl solution in culture medium.

Two independent experiments were conducted with incubation times of 4 or 24 hours with test and control substances. After these periods, cells were washed with sterile PBS at 37°C, then treated with an ethidium bromide monoazo dye solution (Thermo-Fisher Scientific, E1374) at 8.5 µg/mL in PBS supplemented with 2% fetal bovine serum. Plates were exposed to a blue LED light source (440-450 nm, 30W) for 30 minutes for photoactivation, which irreversibly associated with DNA from non-viable cells. This procedure efficiently labeled cells to exclude from nucleus and micronucleus counts and provided a cytotoxicity measure. Cells were then washed with PBS with 2% fetal bovine serum and centrifuged to remove free dye.

Two lysis steps were performed to release nuclei and micronuclei and label DNA. The first lysis used a solution of sodium chloride (0.854 mg/mL), sodium citrate (1 mg/mL), and IGEPAL (0.3 µL/mL) with SYTOX Green fluorescent dye (0.8 µM, Thermo-Fisher

Scientific, S7020). After lysis for 60 minutes at 37°C, plates were centrifuged and received a second lysis solution (sucrose 85.6 mg/mL, citric acid 15 mg/mL, and SYTOX Green 0.4 µM). The lysis solutions were supplemented with 5 µL/well of fluorescent latex beads (AccuCheck Counting Beads, Molecular Probes). After 30 minutes at room temperature, the plate was read on a flow cytometer (Accuri C6, BD Biosciences).

Visual verification of events was performed using a fluorescence microscope (Nikon ECLIPSE TS 100) with a Lumencor® Mira Light Motor (4-NII-FA) and filters with excitation and emission spectra of 460-490 nm and 500-560 nm.

The analysis followed the methodology described in the literature [22]. Briefly, events marked with EMA were excluded from the total count. The events with SYTOX were evaluated according to their size (FSC) and fluorescence (FL1) for discrimination between nucleus and micronucleus. At least 20,000 closed events in the nucleus region were counted in each sample. Data were collected from two independent experiments in eight times as percentage of positive events for EMA and MN positive events for SYTOX and the results were given as fold changes as compared to control wells (untreated cells). The NBP index (Nuclei-to-bead ratios) was used to find differences between the cell division ratios.

Events labeled by SYTOX and EMA were associated with cell death and therefore provided a measure of cytotoxicity. Events labeled only by SYTOX, both nuclei and micronuclei, were evaluated in genotoxicity analysis. Percentages of micronucleus in relation to total SYTOX+ / EMA- events were used as a measure of genotoxicity. The SYTOX + / EMA- beads and nuclei counts provided the Nuclei-to-bead ratio, which is the measure of cell proliferation in this experiment.

Statistical analysis

After reading the equipment (BD CSampler - C6 Flow Cytometer) the data were analyzed and through the program GraphPad Prism 7.0 the results were compared using ANOVA and Bonferroni post-tests.

3. RESULTS AND DISCUSSIONS

Ames Test

According to OECD 471, five strains are used in the Ames test: TA1535, TA98, TA100, TA1537, and *PW2*uvrA. Our study differs from previous studies, such as those by Sommers and Mackay (2005), in several aspects. These authors did not evaluate the effects on *PW2uvrA* and TA102, nor did they test the compound 2-tDCB [23]. Marchioni et al. (2004) also did not evaluate 2-tDCB in TA97, TA98, and TA100 strains and used ethanol as a solvent [24]. Gadgil and Smith (2004) tested 2-DCB at higher concentrations but did not evaluate 2-tDCB and used 384-well plates, a method not regulated by the OECD [25]. Our study utilized the five bacterial strains suggested by the OECD, with concentrations based on the average human consumption of irradiated foods. We used DMSO as a solvent and tested 2-dDCB and 2-tDCB at realistic concentrations.

The detail of the reverse mutation tests in bacteria for 2-dDCB and 2-tDCB, with lines TA-98, TA-100, TA-1535, TA-1537 and *PW2* uvra are found in TAB 3 with metabolic activation and TAB 4 without metabolic activation.

Table 3 - Test in five bacterial cell lines treated with 2-Dodecylciyclobutanone (2-dDCB) and 2-Tetradecylcyclobutanone (2-tDCB) WITH S9 mix

Compound - 2-dDCB		Control +	**Dose 1** 0,83mg/ plate		**Dose 2** 0,27mg/ plate	**Dose 3** 0,09mg/ plate	DMSO [b]
TA-98	Plate 1	1.736	79		62	78	64
2-Aminoanthracene -	Plate 2	2.112	96		67	43	55
5µg/plate [a]	Plate 3	2.096	87		42	85	102
	Average	1.981	87		57	69	74
TA - 100	Plate 1	2.776	157		124	133	123
Sodium Azide - 5µg/plate [a]	Plate 2	1.448	108		167	67	126
	Plate 3	2.032	-	(c)	121	96	115
	Average	2.085	88		137	99	121
TA-1535	Plate 1	52	12		7	4	6
Sodium Azide - 5µg/plate [a]	Plate 2	43	15		9	10	4
	Plate 3	44	11		8	11	17
	Average	46	13		8	8	9

Compound - 2-dDCB		Control +	Dose 1 0,83mg/ plate		Dose 2 0,27mg/ plate		Dose 3 0,09mg/ plate	DMSO [b]
TA-1537	Plate 1	88	18		6		13	6
ICR - 191 Acridine -	Plate 2	227	10		11		11	5
1µg/plate [a]	Plate 3	205	10		7		9	4
	Average	173	13		8		11	5
WP2-*urvA*	Plate 1	227	0	(d)	43		31	36
4-Nitroquinoline-N-oxide -	Plate 2	175	0	(d)	14		15	30
5µg/plate [a]	Plate 3	257	0	(d)	-	(c)	33	28
	Average	220	0		19		26	31
Compound - 2-tDCB								
TA-98	Plate 1	1.736	76		80		72	64
2-Aminoanthracene -	Plate 2	2.112	66		78		61	55
5µg/plate [a]	Plate 3	2.096	54		84		72	102
	Average	1.981	65		81		68	74
TA - 100	Plate 1	2.776	113		120		114	123
Sodium Azide - 5µg/plate [a]	Plate 2	1.448	107		124		136	126
	Plate 3	2.032	143		100		134	115
	Average	2.085	121		115		128	121
TA-1535	Plate 1	52	8		6		6	6
Sodium Azide - 5µg/plate [a]	Plate 2	43	8		18		9	4
	Plate 3	44	8		2		14	17
	Average	46	8		9		10	9
TA-1537	Plate 1	75	9		7		16	4
ICR - 191 Acridine -	Plate 2	212	9		5		5	1
1µg/plate [a]	Plate 3	192	6		5		2	3
	Average	160	8		6		8	3
WP2-*urvA*	Plate 1	227	0	(d)	28		24	36
4-Nitroquinoline-N-oxide -	Plate 2	175	0	(d)	52		26	30
5µg/plate [a]	Plate 3	257	0	(d)	-	(c)	37	28
	Average	220	0		27		29	31

(a) Positive Control compound; (b) Negative Control ; (c) No growth was observed in the colonia; (d) Did not presented reversal even after 48h of regular incubation time

Table 4 - Test in five bacterial cell lines treated with 2-Dodecylciyclobutanone (2-dDCB) and 2-Tetradecylcyclobutanone (2-tDCB) **WITHOUT** S9 mix

Compound - 2-dDCB		+ Control	Dose 1 0,83mg /plate		Dose 2 0,27mg/plate		Dose 3 0,09mg/plate	DMSO [b]
TA-98	Plate 1	1.896	59		67		45	65
2-Nitroflourene - 10µg/plate [a]	Plate 2	1.840	55		63		45	69
	Plate 3	3.128	53		62		67	56
	Average	2.288	56		64		52	63
TA - 100	Plate 1	1.816	136		117		138	65
Sodium Azide - 5µg/plate [a]	Plate 2	2.104	104		165		138	69
	Plate 3	2.496	124		141		166	56
	Average	2.139	121		141		147	63
TA-1535	Plate 1	1.152	27		23		20	17
Sodium Azide - 5µg/plate [a]	Plate 2	1.536	19		13		15	9
	Plate 3	1.248	8		12		14	14
	Average	1.312	18		16		16	13
TA-1537	Plate 1	211	9		7		8	11
ICR - 191 Acridine - 1µg/plate [a]	Plate 2	129	9		6		6	6
	Plate 3	123	8		-	(c)	7	2
	Average	154	9		4		7	6
WP2-*urvA*	Plate 1	1.840	0	(d)	35		34	33
4-Nitroquinoline-N-oxide -	Plate 2	1.872	0	(d)	26		30	25
5µg/plate [a]	Plate 3	1.850	0	(d)	-	(c)	36	17
	Average	1.854	0		20		33	25
Compound - 2-tDCB								
TA-98	Plate 1	1.896	56		75		49	65
2-Nitroflourene - 10µg/plate [a]	Plate 2	1.840	56		63		77	69
	Plate 3	3.128	69		69		61	56
	Average	2.288	60		69		62	63
TA - 100	Plate 1	1.816	112		121		108	155
Sodium Azide - 5µg/plate [a]	Plate 2	2.104	103		111		75	210
	Plate 3	2.496	136		141		103	95
	Average	2.139	117		124		95	153
TA-1535	Plate 1	1.152	19		14		12	17
Sodium Azide - 5µg/plate [a]	Plate 2	1.536	8		16		9	9
	Plate 3	1.248	26		14		6	14
	Average	1.312	18		15		9	13

Compound - 2-dDCB		+ Control	Dose 1 0,83mg /plate		Dose 2 0,27mg/plate		Dose 3 0,09mg/plate	DMSO [b]
TA-1537	Plate 1	189	5		6		9	8
ICR - 191 Acridine - 1µg/plate [a]	Plate 2	120	6		3		3	1
	Plate 3	111	8		7		6	1
	Average	140	6		5		6	3
WP2-urvA	Plate 1	1.840	0	(d)	34		53	33
4-Nitroquinoline-N-oxide -	Plate 2	1.872	0	(d)	8		44	25
5µg/plate [a]	Plate 3	1.850	0	(d)	-	(c)	41	17
	Average	1.854	0		14		46	25

(a) Positive Control compound; (b) Negative Control; (c) No growth was observed in the colonia; (d) Did not presented reversal even after 48h of regular incubation time

Petri dishes containing bacterial strains from *Salmonella Typhimurium* and *E. Coli* were exposed to 2-tDCB and 2-dDCB compounds at 3 different concentrations with and without addition of the S9 fraction. The numbers of revertant colonies induced by the evaluated compounds were compared to a (+) and (-) control and then the mutagenic potential was determined.

No statistically significant data were observed in the reversion rate for 2-dDCB and 2-tDCB at the exposure concentrations tested. Regression rates of negative controls were presented according to the historical averages of other laboratories for the TA-98 cell line [17, 19]. Positive controls had significantly higher reversion rates than those exhibited by negative controls. No evidence of precipitation or toxicity was observed at the concentrations used.

Micronucleus Assay

The studies conducted up to the beginning of this research, aimed at evaluating the toxicity of 2-ACBs, were exclusively performed on colon cells, both of animal and human origin. Therefore, hepatic cell lines were selected to investigate the effects of 2-ACBs in experimental in vitro studies, considering that the liver is frequently impacted by lipid accumulation, and it is well-documented that part of the ingested 2-ACBs is stored in adipose tissues. Additionally, the liver plays a crucial role in the digestive process.

The fundamental and significant difference between the comet assay and the micronucleus test lies in their ability to distinguish between reparable and irreparable damage. The micronucleus test, by its nature, has higher specificity as it identifies exclusively the damage that will not be repaired in the future, unlike the comet assay.

In preliminary studies, our group conducted a pilot assay using the micronucleus technique on a single cell line (HepG2), employing relatively high doses (447, 1422, and 2235 μM) of 2-dDCB and 2-tDCB compounds. No evidence of genotoxicity was observed at these concentration levels, despite the fact that these doses exceed what would normally be ingested by humans in a regular diet. For this reason, our subsequent tests focused on lower concentrations, more aligned with typical human consumption levels [26].

In the micronucleus tests that were performed in this research, was used BRL3A (normal mouse liver), HepG2 (human hepatoma cell) and HTC (rat hepatoma cell) lines that received treatments with the 2-dDCB and 2-tDCB, at concentrations of 100, 300 and 500 μM, during incubation periods of 4 and 24 hours, no genotoxic damage was observed in the evaluated parameters.

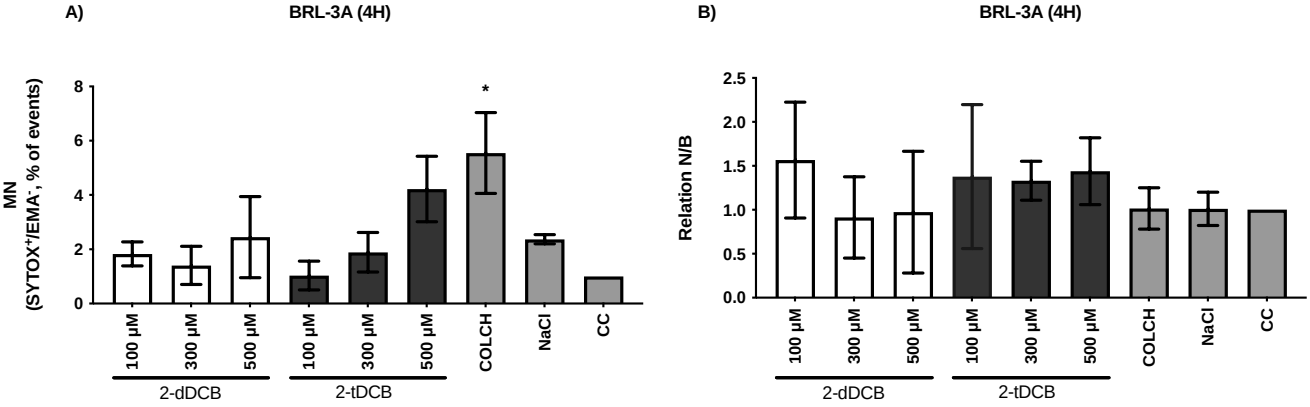
The MN assay met the expectations, showing no significant genotoxic effect in the 3 cell lines studied with the doses and time evaluated.

BRL3-A Cell line

In 4 hours, incubation period, we can observe the effects of compounds and controls in the BRL 3A line in FIG 1 (A). No considerable genotoxic damage was observed at the evaluated concentrations of 100, 300 and 500 μM of both compounds.

Possible changes in the cell cycle rhythm are demonstrated in FIG. 1 (B).

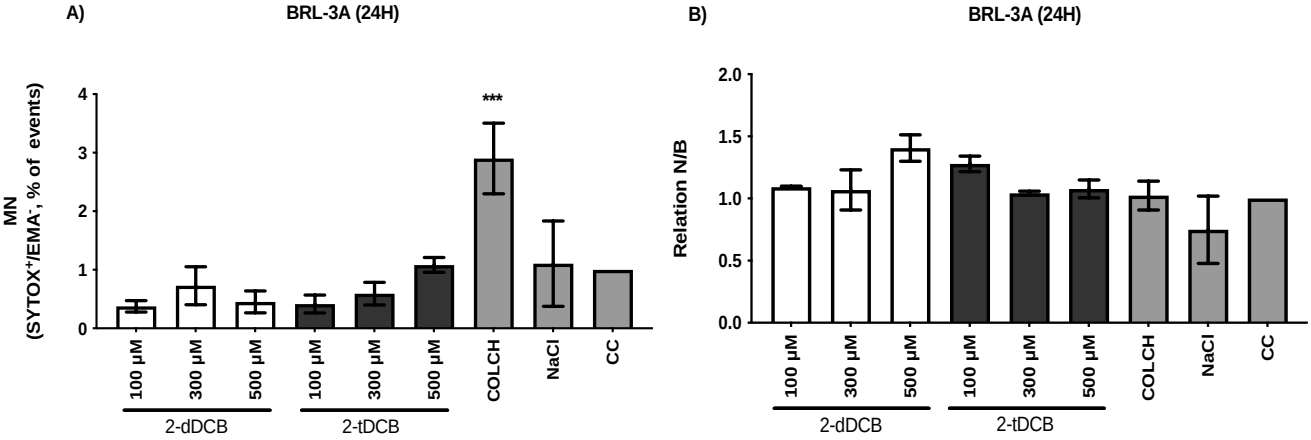
Figure 1 - Possible changes in the cell cycle rhythm



(A) MN formed after cell line receive a treatment with the 2-dDCB and 2-tDCB compounds at the concentrations of 100, 300 and 500 µM. Colchicine, NaCl and CC were used as con-trols (Values expressed in%). **(B)** Bead-core ratio in the BRL 3A cell line exposed to 4 hours with the 2dDCB or 2-tDCB compounds (100, 300 and 500 µM).

The same procedure was adopted for the 24 hours incubation period, including the same parameters and controls. Likewise, no genotoxic effect was observed in both compounds - FIG 2 (A). In FIG. 2 (B) possible changes in the cell cycle rhythm.

Figure 2 - Possible changes in the cell cycle rhythm



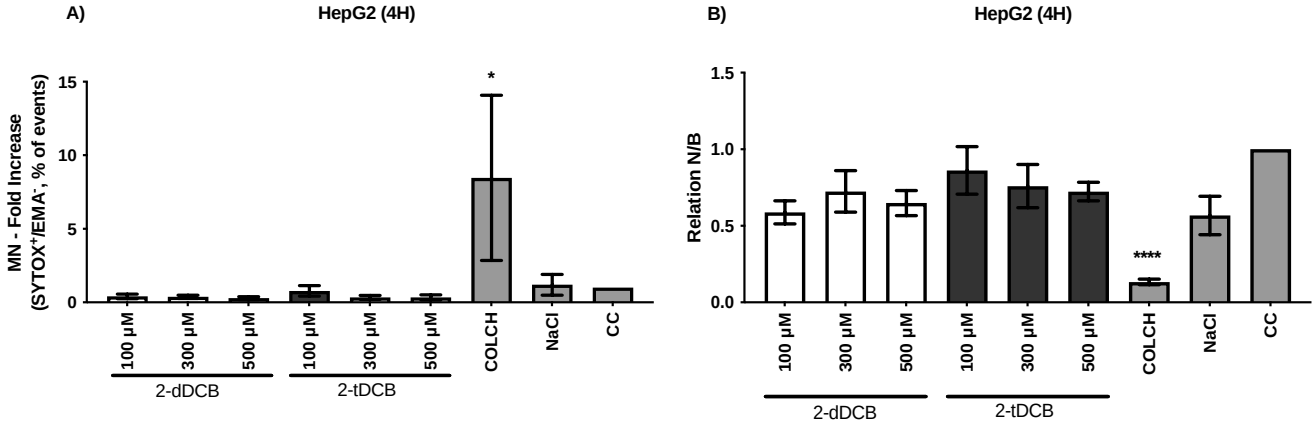
A) MN cells formed after the BRL 3A line were treated with the 2-dDCB and 2-tDCB compounds at the concentrations of 100, 300 and 500 µM. colchicine, NaCl and CC were used as controls (Values expressed in%). Incubation period: 24 hours. (***): p <0.001. **(B)** Bead-core ratio in the BRL 3A line exposed to 24 hours with the 2dDCB or 2-tDCB compounds (100, 300 and 500 µM). No changes were observed.

HepG2 Cell Line

Effects of controls and compounds in the HepG2 line for the 4-hour incubation period is observed in FIG. 3 (A). As in the previous cell line evaluated, no considerable genotoxic damage was observed at the evaluated concentrations of 100, 300 and 500 µM both compounds - FIG 3 (A).

Possible changes in cell cycle rhythm are demonstrated in FIG 3 (B).

Figure 3 - Possible changes in the cell cycle rhythm



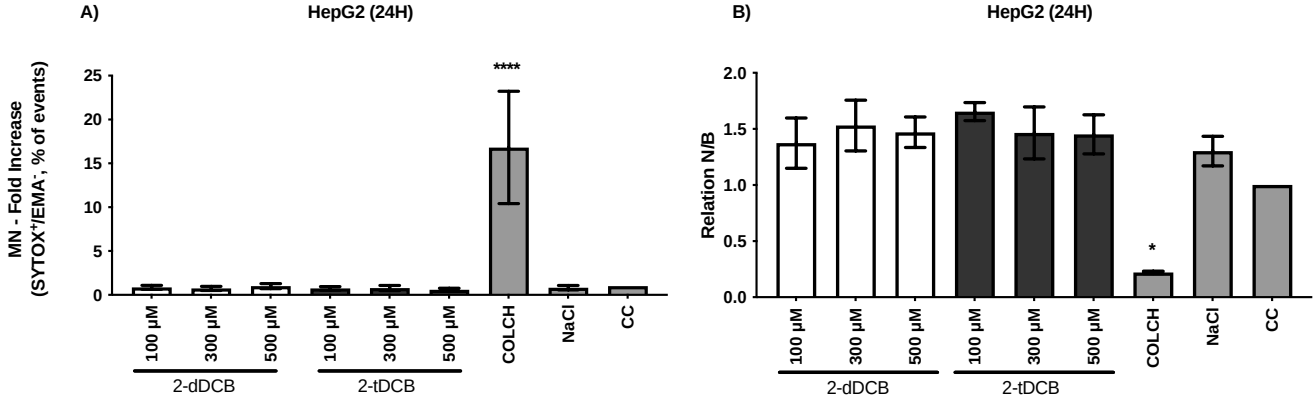
(A) MN formed after the HepG2 cell line received treatment with the compounds of 2-dDCB and 2-tDCB at the concentrations of 100, 300 and 500 µM, incubation period of 4 hours. Colchicine, NaCl and CC (cell control) were used as controls (values expressed in%). (*): $p < 0.05$. **(B)** Bead-core ratio in HepG2 line exposed at 4 hours with 2dDCB or 2-tDCB compounds (100, 300 and 500 µM). (****): $p < 0.0001$. No changes were observed.

In the same way occurred in the incubation period of 24 hours, using the same parameters and controls determined, again, no effect was observed - FIG 4 (A).

The same standards were followed, and no genotoxic damage was observed at the concentrations evaluated 100, 300 and 500 µM of both compounds - FIG 4 (A).

No changes are observed in the cell cycle rhythm, shown in FIG. 4 (B).

Figure 4 - Possible changes in the cell cycle rhythm



(A) MN formed after the HepG2 cell line received treatment with the compounds of 2-dDCB and 2-tDCB at the concentrations of 100, 300 and 500 µM, incubation period of 24 hours. Colchicine, NaCl and CC (cell control) were used as controls (values expressed in%). (**): $p < 0.01$. **(B) -** Bead-core ratio in HepG2 line exposed at 24 hours with 2dDCB or 2-tDCB compounds (100, 300 and 500 µM). (*): $p < 0.05$. No changes were observed.

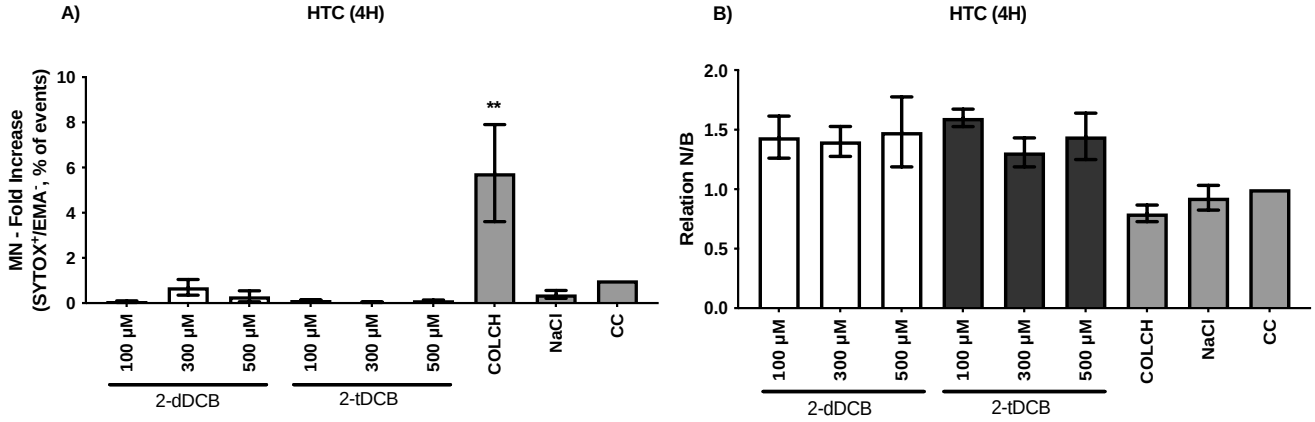
HTC Cell Line

Once again, another cell line was taken to repeat the tests. This time we evaluated the HTC cell line (cells derived from liver tumors of rats). The same procedures performed in the previous cell lines were adopted, with the same incubation periods, i.e., 4 and 24 hours for both compounds.

FIG. 5 (A) shows the effects of the controls and compounds on the HTC cell line, for the 4-hour incubation period. At the concentrations studied (100, 300 and 500 µM of 2-dDCB and 2-tDCB), no genotoxic damage was observed according to FIG 5 (A).

Changes in cell cycle rhythm were not noticed - FIG 5 (B).

Figure 5 - Possible changes in the cell cycle rhythm



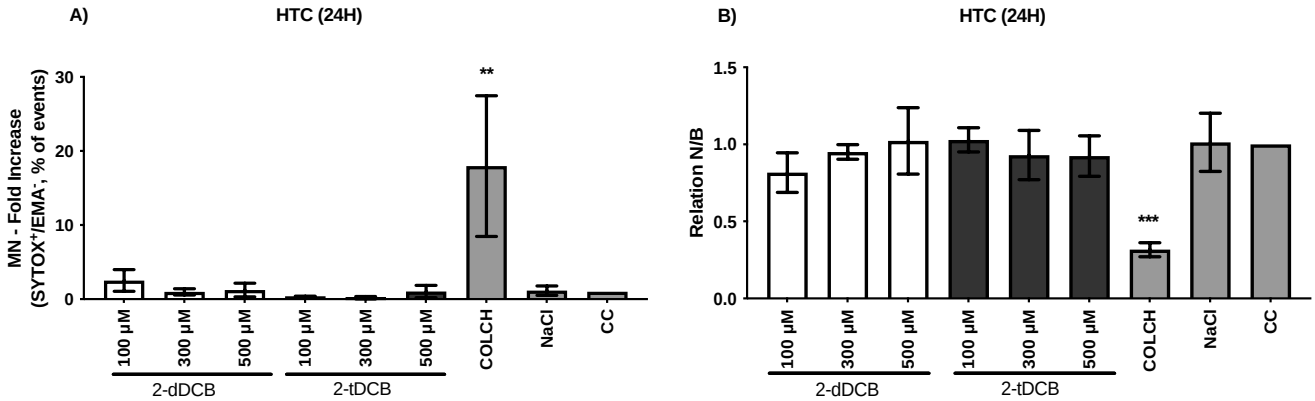
(A) MN formed after the HTC line receiving treatment with the compounds of 2-dDCB and 2-tDCB at concentrations of 100, 300 and 500 µM, incubation period of 4 hours. Colchicine, NaCl and CC (cell control) were used as controls (values expressed in%). (**) p <0.05. **(B) -** Bead-core ratio in the HTC cell line exposed to 4 hours with 2dDCB or 2-tDCB compounds (100, 300 and 500 µM). No changes were observed.

Finally, in the 24-hour incubation period, the HTC cell line was exposed to the controls and concentrations of 2ACBs and there was no genotoxic impact caused by the concentrations of test compounds evaluated - FIG 6 (A).

The same standards were followed, and no genotoxic damage was observed at the concentrations of 100, 300 and 500 µM of both compounds - FIG 6 (A).

No changes are observed in the cell cycle rhythm, shown in FIG 6 (B).

Figure 6 - Possible changes in the cell cycle rhythm



(A) MN formed after the HTC cell line receiving treatment with the compounds of 2-dDCB and 2-tDCB at concentrations of 100, 300 and 500 µM, incubation period of 24 hours. Colchicine, NaCl and CC (cell control) were used as controls (values expressed in%). (**) p <0.05. **(B) -** Bead-core ratio in the HTC cell line exposed to 24 hours with 2dDCB or 2-tDCB compounds (100, 300 and 500 µM). (***): p <0.001. No changes were observed.

4. CONCLUSIONS

Food irradiation technology has been widely applied globally. Two factors motivated this study: Although previous studies have evaluated the genotoxic potential of 2-ACBs, none have focused on hepatocytes, crucial cells in the digestive system often affected by lipid accumulation. Considering the increasing incidence of hepatocellular carcinoma related to obesity or with unknown etiology, assessing the genotoxicity of 2-ACBs in the liver is essential. In Brazil, no regulatory agency oversees irradiated foods. Our group investigates the potential adverse effects of these foods on the body, given that 2-ACBs, by-products of irradiated foods, may promote colon adenocarcinoma at certaine concentration. Establishing safe limits for food irradiation is imperative. Our goal is to collect comprehensive data and eventually present it to regulatory agencies to ensure appropriate oversight of the irradiation process and establish safe dose intervals.

In the Ames test, no statistically significant data were observed in the reversion rate for 2-dDCB and 2-tDCB at the tested concentrations. Positive controls showed significantly higher reversion rates than negative controls, with no evidence of precipitation or toxicity.

In the micronucleus assay, few studies evaluate the genotoxic effects of 2-ACBs. Most used the comet assay, except for Yamakage et al. (2014), who used the micronucleus test in bone marrow, while our study utilized hepatocytes. Previous studies focused only on colon cells. We chose hepatic cell lines due to the accumulation of 2-ACBs in adiposes tissues and the liver's direct relationship with the digestive process.

The micronucleus test, which detects irreparable damage, unlike the comet assay, was conducted with BRL3A, HepG2, and HTC cells treated with 2-dDCB and 2-tDCB at concentrations of 100, 300, and 500 μM for 4 and 24 hours, with no genotoxic damage observed. The MN assay results showed no significant genotoxic effect at the evaluated doses and times.

Although the results did not show mutagenic or genotoxic effects at the tested concentrations, further *in vitro* and *in vivo* tests are necessary to ensure that 2-ACBs consumption does not cause health damage.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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