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IN VIVO GENOTOXICITY OF 2-ALKYLCYCLOBUTANONES IN LIVER CELLS FROM RATS FED WITH IRRADIATED COCOA BUTTER USING FLOW CITOMETRY

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ABSTRACT

Food irradiation proves to be an effective technique of eliminating some pathogens from food and this has gained significant attention to its potential for food safety. Since 1990, studies on the toxicological safety of 2-Alkylcyclobutanones have been conducted extensively. 2-Alkylcyclobutanones are unique radiolytic products generated by the radiation-induced breakage of triglycerides in food, are exclusively found in irradiated lipid containing foods. 2-dodecylcyclobutanone (2-dDCB) and 2-tetradecylcyclobutanone (2-tDCB) are the predominant compounds detected in irradiated food. Despite studies showing non-genotoxicity of 2-ACBs (2-Alkylcyclobutanones), the results are conflicting and therefore we continue the studies in order to confirm the compounds safety for human health. In vivo micronucleus test were performed to verify the 2-ACBs genotoxic effects in hepatic cells using flow citometry. We used cocoa butter irradiated with 20 kGy at IPEN GAMACELL. A group with animals (IPEN Ethical Animal Experimentation Committee, process number 148/14) was treated with daily intake of irradiated cocoa butter, synthesized 2-Dodecylcyclobutanone and 2-Tetradecylcyclobutanone for two months. Hepatic cells were selected for genotoxicity analysis due to the liver importance in the compounds metabolization. Analyzes were made by micronucleus test with specific cells extracted from hepatic tissue using flow cytometry, which is an alternative to conventional techniques, allowing faster analysis and reduction in the animals number that is a subject much approached in research today. The improvement of the analytical techniques is important for the research future since the irradiation process is already consolidated. The results confirmed the safety of the food irradiation process, as they did not indicate the genotoxic potential of the samples.

1. INTRODUCTION

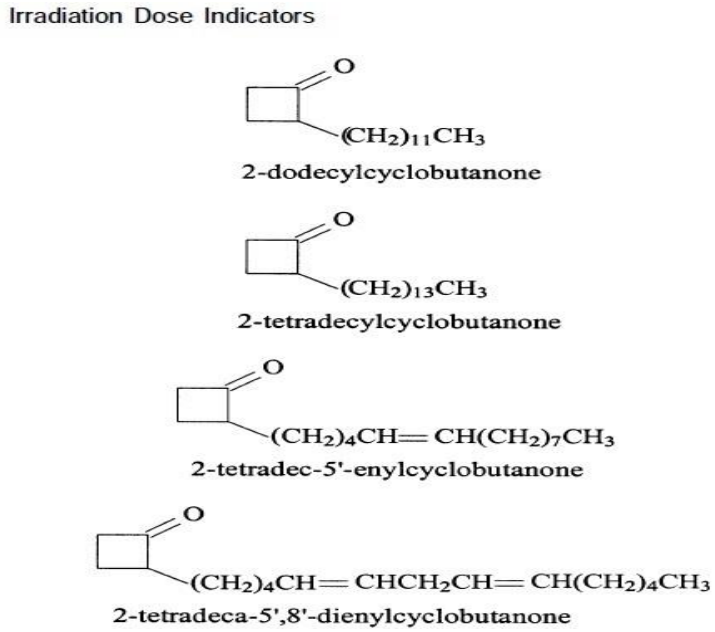
1.1 Food irradiation

Food irradiation has been demonstrated as an effective tool and gained great attention due to its potential for food safety assurance, reducing microbial load, insect pest control in agricultural products, extension of the period of shelf, budding inhibition [1]. The critical factor in this process is how to apply precise radiation doses to meet the requirement of the process without causing significant losses of sensory and nutritional values to foods [2]. However, there are some limitations as well: the formation of radiolytic compounds such as 2- Alkylcyclobutanones that are derived from the irradiation of foods containing fat [3].

1.2 2-Alkylcyclobutanones formation

Since 1990, studies on the toxicological safety of 2-Alkylcyclobutanones have been conducted extensively [4]. 2-Alkylcyclobutanones are unique radiolytic products generated by the radiation-induced breakage of triglycerides in food, are exclusively found in irradiated lipid containing foods[5],2-dodecylcyclobutanonefrom radiolysis of palmiticacid (2-dDCB) and 2-tetradecylcyclobutanone from stearic acid (2-tDCB),are the predominant compounds detected[6]. They have the same number of carbons (n) as their fatty acid precursors, with an alkyl chain of (No -4) carbons in ring position 2 [7] Fig.1. and are thus considered unique markers of food irradiation[8].
The toxic potential of 2-ACBs has been previously investigated by a French–German research group in an EU interregional programme [9], in which the tumour-promoting potential of 2-tDCB and 2-(tetradec-5_-enyl)-cyclobutanone (2-tDeCB) was reported [10]. Despite studies showing non-genotoxicity of 2-ACBs, the results are conflicting and therefore we continue the studies in order to confirm the compounds safety for human health. In vivo micronucleus test were performed to verify the 2-ACBs genotoxic effects in hepatic cells using flow citometry. However, furtherstudies are required to resolve remaining uncertainties regarding the genotoxicity of 2-ACBs, because very few studies have been conducted to investigate the genotoxicity of pure 2-ACBs according to specific test guidelines [11].

Figure 1: Structure of the basic side chains of the four most common Alkylcyclobutanones found in foods containing lipids irradiated



(Gadgil; Hachmeister; Smith et al. 2002)[12]

2. MATERIAL AND METHODS

2.1 Samples

Cocoa butter samples containing 29% palmitic acid (2-Dodecylcyclobutanone precursor) and 32% stearic acid (2-Tetradecylcyclobutanone precursor) were used [13]. It was gamma-irradiated (20 kGy, dose rate 0.473 kGy/h) using a ^{60}Co source (IPEN Gammacell 220) at room temperature. They were fluidized in a water bath at 38ºC and administered orally daily to all animals 1 mL/kg/bw.

2.2 Chemicals

Type I Collagenase (CAS N.9001-12-1 Sigma Aldrich®) was solubilized (300 μg/mL) in Hank's Balanced Salt Solution (HBSS). Cyclophosphamide monohydrate (CP) (CAS N.6055-19-2 Sigma Aldrich®) was solubilized in saline solution (NaCl 0,9%). Cell suspensions were properly incubated with SYTOX® Green (Molecular Probes, S7020) and ethidium monoazide bromide (EMA, Molecular Probes, E1374) to label nuclear and micronuclear DNA (SYTOX) and to discriminate nuclei from dead cells (EMA).

2.3 Animals housing conditions

Groups of 10 male *Wistar* rats were bred and maintained for two months at IPEN Animal Facility under all principles of animal welfare (12/12 h day night cycles, room temperature: 22-23 ºC, 44-65% relative atmospheric humidity, no more than three animals/cage), as specified by IPEN Ethical Animal Experimentation Committee (process number CEUA-IPEN Nº 148/14), including food and water *ad libitum* availability. Two animals were supplemented with daily oral dose of 1g of irradiated cocoa butter containing 2-alkylcyclobutanones, two animals were treated with daily oral dose of 4 mg / kg of 2-Dodecylcyclobutanone and 2 animals with the same dose of 2-Tetradecylcyclobutanone dissolved in oil for 5 days. Positive controls for genotoxicity were represented by 2 animals injected intraperitonially with cyclophosphamide (50 mg/kg/bw), 24 hours prior the experiment. Negative controls were represented by 2 animals injected with 1 mL of vehicle control (NaCl 0,9 %) and more two animal without treatment and interferences, according to OECD standards, wich are a unique tool for assessing the potential effects of chemicals on human health and the environment.

2.4 Flow Cytometry

2.4.1. Hepatic cell isolation

Animals were euthanatized by exposure to CO_2 approximately 24 h following the last treatment. Livers were exposed and fragments of 3 to 4 mm^3, were dissociated passing through syringe needle in HBSS with collagenase (200 μg/mL). Tissue pieces were incubated at 37 ºC for 1 hour, with vigorous shaking every 15 minutes. Suspensions were centrifuged (1500 rpm, 5 min, RT) and cell pellets were suspended in ammonium chloride (15.2 mM in water) to lyse erythrocytes and kept on ice for 5 minutes. Cells were centrifuged as described and suspended in ice-cold phosphate-buffered saline solution (PBS).

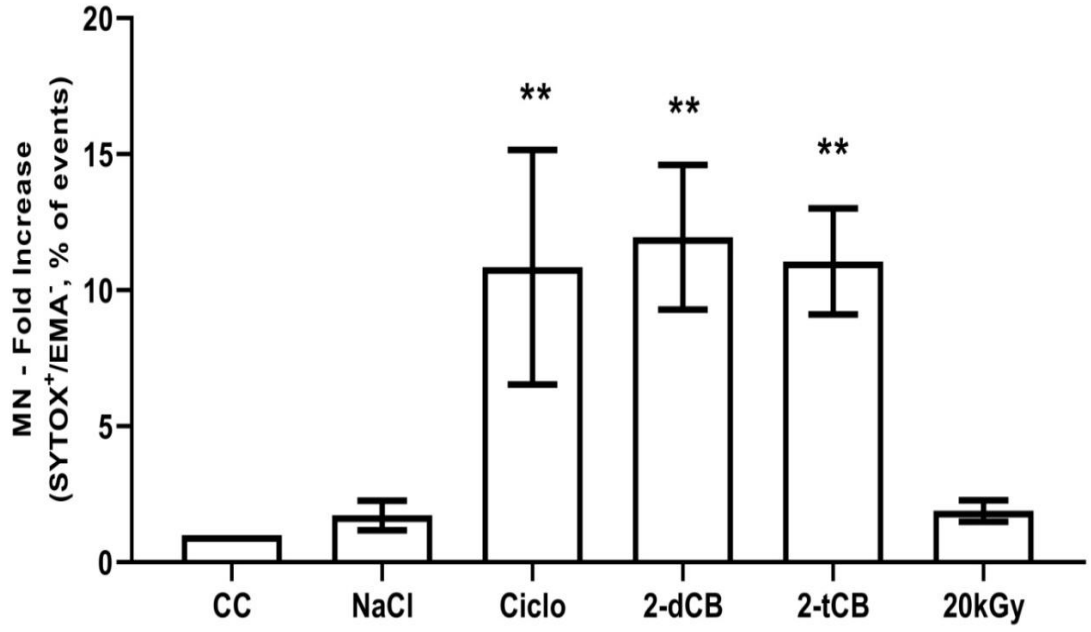
2.4.2. Flow cytometric analysis

Isolated liver cells from rats were plated (100 μL/well) in 96-well plates in quadruplicates. Briefly, cells were incubated with EMA (5 μg/mL) and exposed to blue led light (30 W) in an ice bath to photoactivaction of dye. Plates were centrifuged (1500 rpm, 15 min, RT) and cells were lysed with buffer [14].

3. RESULTS

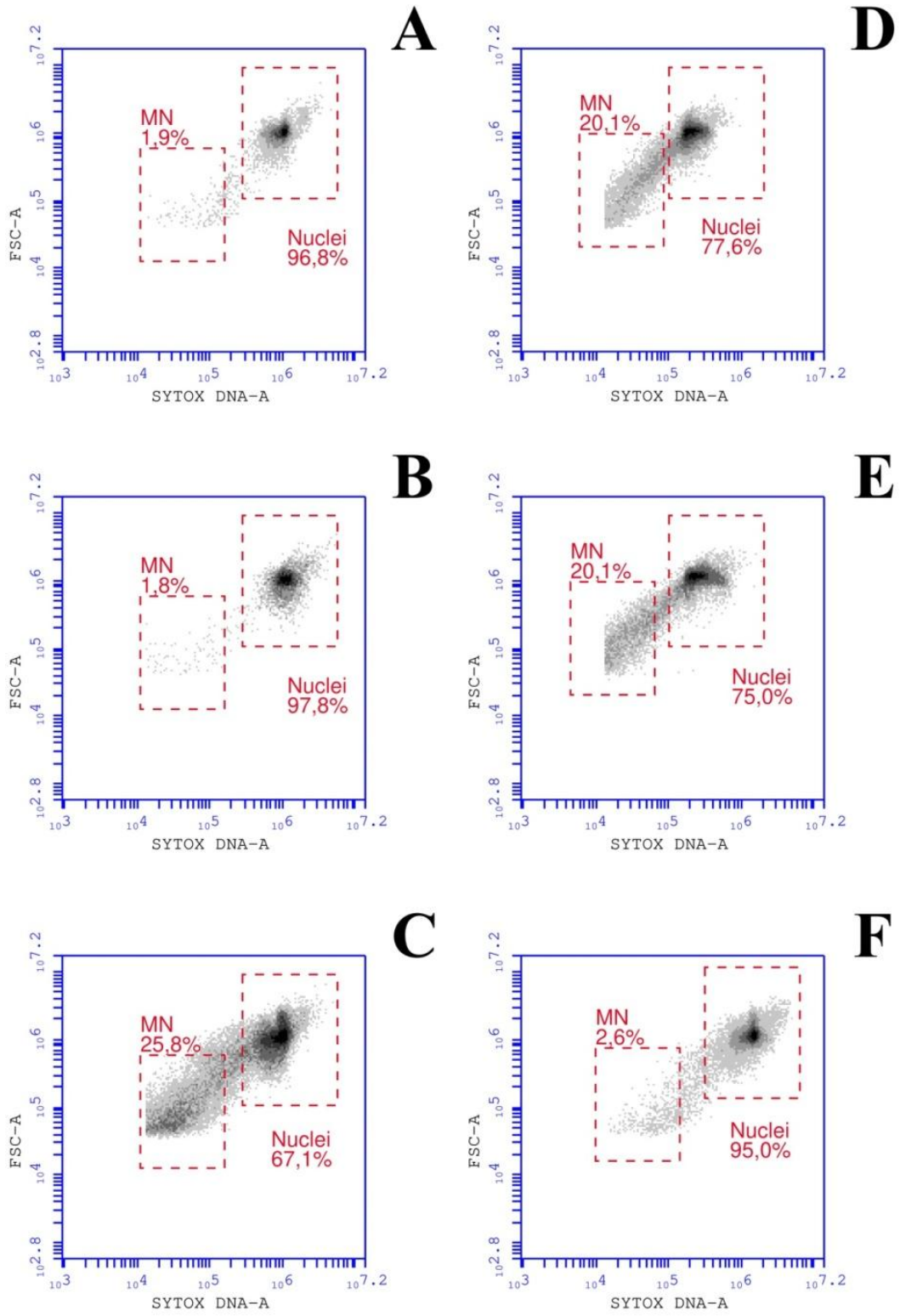
Body weight and food intake in each group were similar throughout the experimental period. We administered 2-dDCB or 2-tDCB to the mice twice orallyfor 5 days. Statistically significant increases were observed in the frequency of micronucleous (MNs) after doses of 4 mg/kg/bw day of 2-dDCB and 2-tDCB. There were significant differences between the 2-dDCB or 2-tDCB groups and the vehicle control group. On the other hand, the group that ingested the irradiated cocoa butter, representing the amount naturally ingested by food, did not present significant amounts that induce genotoxicity (Graf.1). The chemical positive control (CP) clearly induced MNs(Fig.2).

Graphic 1: Percentage of MN (% of total events) of liver cells of rats



CC (cellular control), NaCl (Negative control), Ciclophosphamide (Positive control), 2-Dodecylcyclobutanone, 2-Tetradecylcyclobutanone and 20 kGy irradiated cocoa butter. Bars: SEM (Standard error of means). (**): $p < 0,01$ (difference from negative control).

Figure 2: Representative Flow Citometry of Experimental examples from group results.



(A) Cells control,(B) NaCl 0,9%; (C) Ciclophosphamide, (D) 2-Dodecylcyclobutanone, (E) 2-Tetradecylcyclobutanone and (F) 20 kGy Irradiated cocoa butter.

4. DISCUSSION AND CONCLUSION

According to the literature, few in vivo studies have been performed to evaluate the genotoxicity of 2-ACBs.
In 2002, Horvatovich et.al [15], reported that less than 1% of 2-ACBs are excreted in feces and a small amount was detected in adipose tissue.
Raul et.al 2002[10], affirmed the promotion of colon cancer, causing the scientific community to perform more studies on the subject. Until then studies using micronuclei had not been performed.
Yamakage et.al 2014[11] performed the bone marrow micronucleus test and suggested the non-genotoxicity of 2-ACBs.
The last micronucleus test performed by Beom S.Song et.al 2018[16], uses doses of 250, 500, 1000 and 2000 mg/kg/bw and has results in line with Yamakage et.al 2014. But compared to the present article there is divergence in the results when it comes to micronucleus analysis in specific target organ cells.
Previous studies used bone marrow, while this study used cells extracted from liver tissue, which reported an increase in the number of MN. The rat liver micronucleus test yields positive results with complex metabolic transforming substances, which negative results are obtained in bone marrow MN, metabolites produced in the liver do not reach the bone marrow. This technique using rat liver removes the problem of specificity by studying different target organs, according Cliet et al 1989 [17].
We conclude that the consume of 2-ACBs through food in this study represented by irradiated cocoa butter has no genotoxic potential. Specific cell analyzes obtained satisfactory results indicating that they are more appropriate than bone marrow cell analyzes for certain compounds. Target organ studies such as pancreas, small intestine are indicated for analysis as they participate in fat metabolization and absorption, unstable mutagenic compounds may not be detectable in bone marrow MN.

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