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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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1. Introduction

Food irradiation, recognized for its efficacy and safety in food preservation, effectively combats a wide range of microorganisms, facilitating pest disinfestation and extending shelf-life without chemical by-products [1]. Utilizing gamma radiation, this process exerts both direct and indirect effects on treated materials, chiefly by halting microorganism cell division through various molecular alterations, including DNA fragmentation and oxidation, thereby reducing microbial content [2]. Despite its benefits in food hygiene and spoilage reduction, concerns have emerged regarding the potential formation of 2-Alkylcyclobutanones (2-ACBs) in fat foods under high-dose irradiation, substances linked to potential genotoxic effects [3]. Initial investigations into 2-ACBs' cytotoxicity on liver cells [4] have prompted further analyses to unravel their genotoxic and mutagenic impacts, employing methodologies like the Ames test and micronucleus frequency assay [5, 6, 7]. This growing body of research underscores the critical need for comprehensive evaluations of 2-ACBs, aiming to address public health concerns related to the consumption of irradiated foods. As such, this study endeavors to illuminate the mechanisms through which 2-ACBs may exert tumorigenic effects, providing essential insights into their safety profile and contributing to the broader understanding of food irradiation's implications on health [8- 16].

2. Methodology

Tested Substances: In this study, we used the compounds 2-dDCB (Dodecylcyclobutanones) and 2-tDCB (Tetradecylcyclobutanones), both with purity confirmed above 99%, supplied by Sigma-Aldrich. For the Ames test, DMSO (Dimethyl Sulfoxide) was chosen as the solvent due to its specific suitability for this assay. For the micronucleus assays, ethanol at a final concentration of 2% was used, considering the compatibility of this solvent with the procedure. This distinction in solvent choice reflects the specific needs of each type of assay conducted.

Ames Test: We adopted the protocol of Yamakage et al. [16] with modifications to solubilize the 2-ACBs, applying specific concentrations that had shown stability in previous studies (0,83, 0,27 and 0,09 mg / plate). The Laboratory of Genotoxicity in Microorganisms conducted the assays, using five distinct bacterial strains for a broad spectrum of mutation detection.

- **Controls and Cell Lines:** The selection of cell lines (TA-98, TA-100, TA-1535, TA-1537 and PW2 uvra) and the negative and positive controls followed international standards, with DMSO as the negative control and specific substances for each bacterial strain as positive controls, adapting to the metabolic activation needs with the use of S9 fraction (The freeze-dried mouse liver homogenate) induced with Aroclor 1254 (Moltox Molecular Toxicology, Inc., Boone, NC 28607, USA).
- **Preparation of Inocula and Culture Media:** Inocula were prepared in Oxoid Nutrient Broth No. 2, adjusting the optical density to ensure assay consistency. The minimal and top agar media were prepared according to specifications, with specific additives to promote bacterial growth and facilitate mutation detection.
- **Statistical Analysis:** Data analysis was performed using the Dunnett-t test, as recommended by scientific societies, to evaluate the statistical significance of the observed mutagenic reversions compared to controls.

Micronucleus Assay: We adapted the protocols of Bemis et al. [17] to examine the genotoxic effects of the 2-ACBs at concentrations of 100, 300 and 500 μM on hepatic cells (HepG2, BRL3A and HTC), preparing solutions of the compounds in specific concentrations and using colchicine as a positive control compared to a standard negative control (0.9% NaCl).

- **Statistical Analysis:** After reading the equipment (BD CSampler - C6 Flow Cytometer) the data were analyzed and through the program GraphPad Prism 9.0 the results were compared using ANOVA and Bonferroni post-tests.

3. Results and Discussion

In this study, we first assessed the mutagenic potential of the compounds 2-dDCB and 2-tDCB using the Ames test with specific bacterial strains, and then we explored their genotoxic potential in liver cell lines.

Ames Test

In the Ames test, we used strains TA 1535, TA98, TA100, TA1537, and WP2uvrA, following OECD 471 guidelines. We observed no significant increase in reversion rates for 2-dDCB and 2-tDCB at the tested concentrations, compared with negative controls and assessed against the historical averages of other laboratories. Positive controls showed significantly higher reversion rates, validating the test's effectiveness. This indicates that, under experimental conditions, 2-dDCB and 2-tDCB did not demonstrate mutagenic potential.

Micronucleus Assay

To illustrate, we selected the micronucleus assay results in the HepG2 cell line, given space limitations for including graphics.

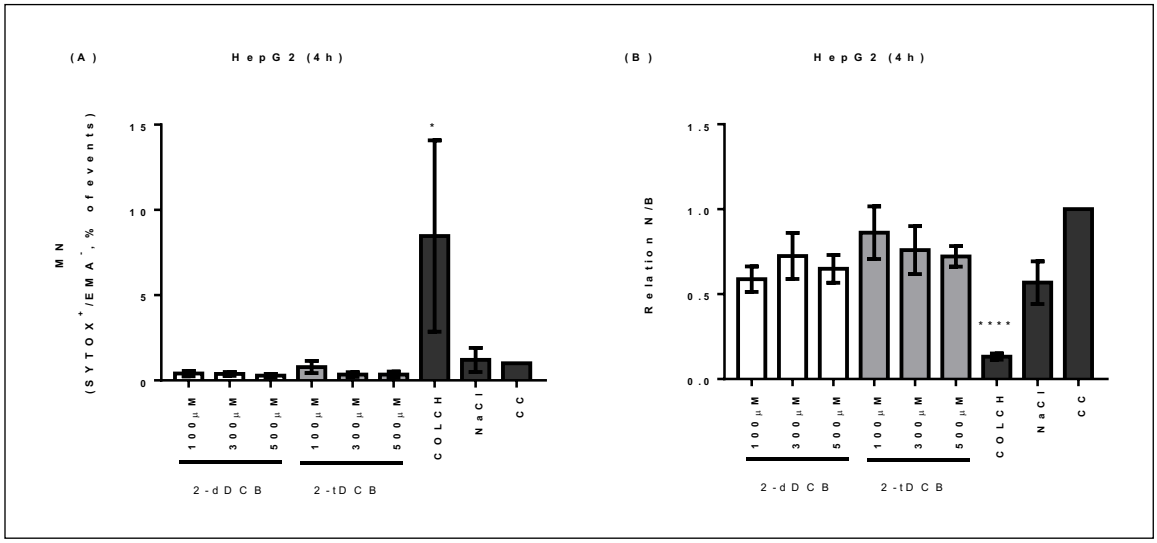

Figure 1: (A) Formation of micronuclei (MN) in the HepG2 cell line after treatment with 2-dDCB and 2-tDCB at concentrations of 100, 300, and 500 µM, for an incubation period of 24 hours. Controls used include colchicine (positive control), NaCl, and cell control (CC), with values expressed as a percentage. (**) indicates $p < 0.01$. **(B)** Nucleus-cytoplasm ratio in the HepG2 line exposed for 24 hours to the compounds 2-dDCB and 2-tDCB (100, 300, and 500 µM). (*) indicates $p < 0.05$. No significant changes were observed.

In the micronucleus assays, we used BRL3A, HepG2, and HTC cell lines treated with 2-dDCB and 2-tDCB at concentrations of 100, 300, and 500 µM for incubation periods of 4 and 24 hours. Colchicine was used as a positive control and DMSO as a negative control. In all cases, no significant genotoxic damage was observed at the evaluated concentrations. The assay met expectations, showing no significant genotoxic effect in the three studied cell lines.

The application of food irradiation technology has been increasing globally, making it crucial to assess the potential genotoxic effects of 2-ACBs. This study highlights the importance of investigating these compounds in hepatic cells, given the liver's role in the digestive system and its tendency to accumulate fat, where some 2-ACBs may be deposited.

Our results showed no mutagenic or genotoxic effects at the tested concentrations, contributing to the safety of irradiated foods containing 2-ACBs. However, we emphasize the need for further research, both *in vitro* and *in vivo*, to ensure that the consumption of 2-ACBs does not cause health harm. We intend to present our data to competent regulatory agencies, aiming to establish safe irradiation dose ranges for food.

4. Conclusions

This study provides robust evidence that the compounds 2-dDCB and 2-tDCB do not exhibit mutagenic or genotoxic potential at concentrations up to 500 µM, as assessed by Ames tests and micronucleus assays in various cell lines, including HepG2, BRL3A, and HTC. The findings reinforce the safety of irradiated foods containing 2-ACBs, significantly contributing to the body of knowledge on food irradiation. However, they underline the need for further research to fully elucidate the long-term effects of consuming these compounds and to assess their interaction with other food components under different irradiation conditions. This study highlights the importance of continuing rigorous food safety evaluations to ensure public health.

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