IN VIVO EVALUATION OF GENOTOXIC POTENTIAL OF LIVER CELLS FROM RATS FED WITH IRRADIATED DIET USING FLOW CYTOMETRY.

R.Martins[a*], L.R. de Carvalho[a], D.P.Vieira[a], A.B.Barbezan[a], A. L. C. H. Villavicêncio[a]

[a] Instituto de Pesquisas Energéticas e Nucleares (IPEN) na Universidade de São Paulo (USP) Av. Prof. Lineu Prestes, 2242, Cidade Universitária, São Paulo/ SP CEP: 05508-000- Brasil

1. PURPOSE

Radiation sources became widely available since 1960's, and between its main uses are the applications in food irradiation and research of effects of ionizing radiation on food products. Despite some public concern, the process is safe, free from chemical residues and presents advantages for preservation and storage. Nevertheless, safety dose parameters must be adopted in irradiation procedures to inhibit formation of undesirable and/or toxic products, for example, 2-ACB's (2-alkylcyclobutanones) that are cyclic compounds containing four carbon rings that can be formed in food when its fat content is irradiated through breakdown of fatty acids. 2-ACBs are considered a unique class of compounds due to divergences between results of its mutagenicity potential collected from different studies. In this study, a cell population collected from rat livers were chosen for *in vivo* genotoxicity analysis because the importance of the liver in the metabolization of compounds. Analysis was performed using the micronuclei test using flow cytometry, allowing faster analysis, use of few materials and reduction in the number of animals, what is a subject much addressed currently in research. Irradiated rat diet did not show any genotoxic effect on liver cell populations. The improvement of the techniques is important for the future of the research since the irradiation process is already consolidated.

2. METHODS

2.1 Chemicals

Normocaloric (about 3% of fat of total dry mass) rat industrial diet (Nuvilab®) was gamma-irradiated (DOSE, TAXA) using a ^{60}Co source (Gammacell 220) at room temperature. Type I Collagenase (CÓDIGO) was solubilized (300μg/mL) in Hank's Balanced Salt Solution (HBSS). Cyclophosphamide monohydrate () and Methyl-Methane Sulphate (MMS,) were diluted 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).

2.2 Animals

Groups of male Wistar rats were bred and maintained until the age of 8 months at IPEN Animal Facility under all principles of animal welfare (12/12h day night cycles, room temperature: 22-23ºC, 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. Five animals were fed after weaning with normocaloric diet irradiated as described above, and three animals were fed with standard autoclaved diet. Positive controls for genotoxicity were represented by 2 animals injected intraperitonially with cyclophosphamide (50mg/kg) and other 2 injected same way with MMS (25mg/kg) 24 hours prior the experiment. Negative controls were represented by 2 animals injected with 1mL of vehicle control (NaCl 0,9%) (OECD).

2.3 Flow Cytometry

2.3.1. Hepatic cell isolation

Animals were euthanatized in a CO_2 chamber. Livers were exposed and fragments of 3 to 4 mm^3 were dissociated passing through syringe needle in HBSS with collagenase (200μg/mL). Tissue pieces were incubated at 37ºC 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 (15.2mM 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).

2.3.2. Flow cytometric analysis

Isolated liver cells from rats were plated (100μL/well) in 96-well plates in quadruplicates. The used protocol for labelling nuclear/micronuclear DNA was described elsewhere (Bryce SM, 2007). Briefly, cells were incubated with EMA (5μg/mL) and exposed to blue led light (30W) in an ice bath to photoactivaction of dye. Plates were centrifuged (1500 rpm, 15min, RT) and cells were lysed with buffer containing SYTOX®-Green (0.4μM) and RNAse-A to avoid RNA labelling. After incubation, (1h, 37ºC, dark) and pre-acquisition steps, cells were analyzed using a Accuri C6 Cytometer (BD Biosciences), through acquisition of fluorescence in FL1 (SYTOX®-Green) and FL3 (EMA) channels. Micronuclei frequencies from groups were compared by Kruskal-Walis method.

Micronuclei were scored as shown in Fig 1. Examples of results were shown on Fig. 2. Results are shown in Fig 3.

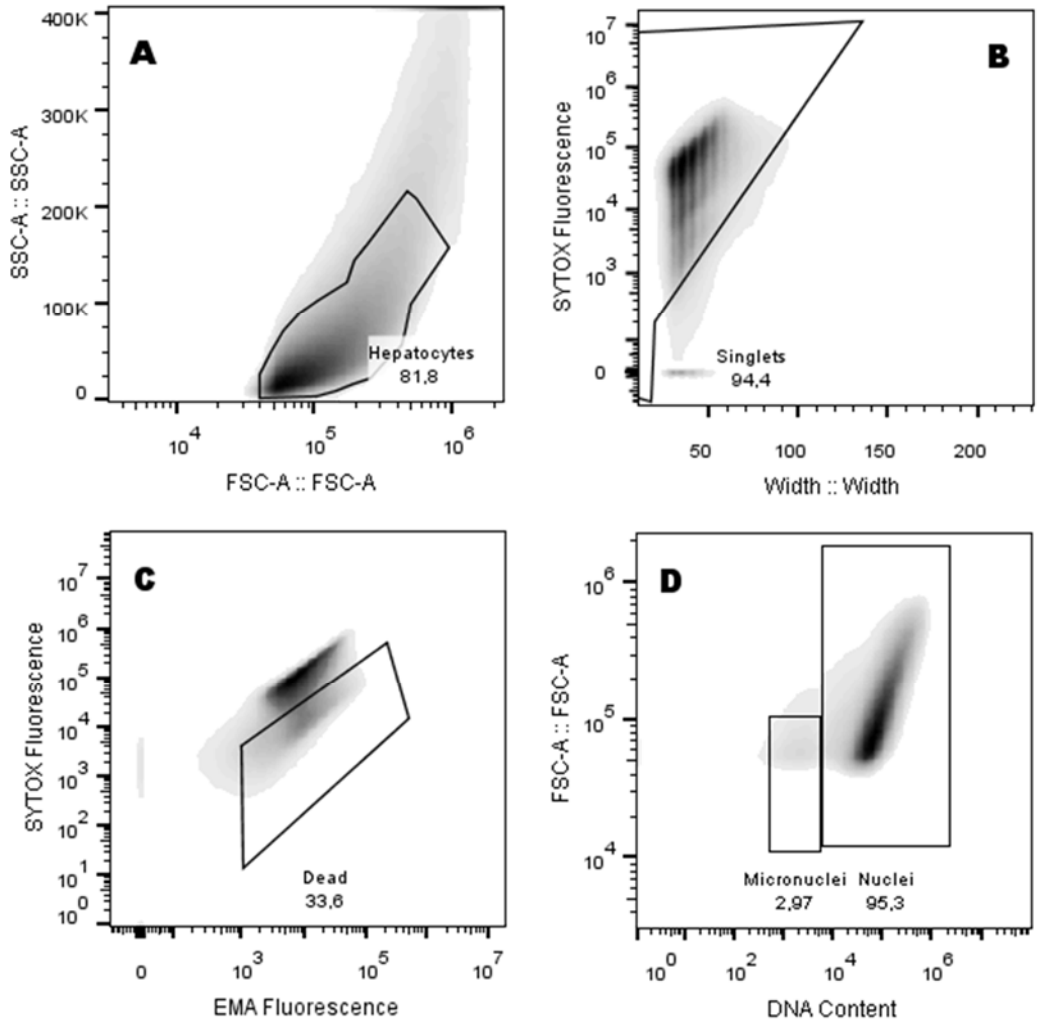

Figure 1: Gating strategy for analysis of micronucleus frequency from experiments. (A): Cell gating; (B): Doublet elimination/singlet characterization; (C) Dead cells exclusion from analysis; (D) Nuclei and MN regions delimited on plot. Numbers on plots refers to percentages of total events depicted on each plot.

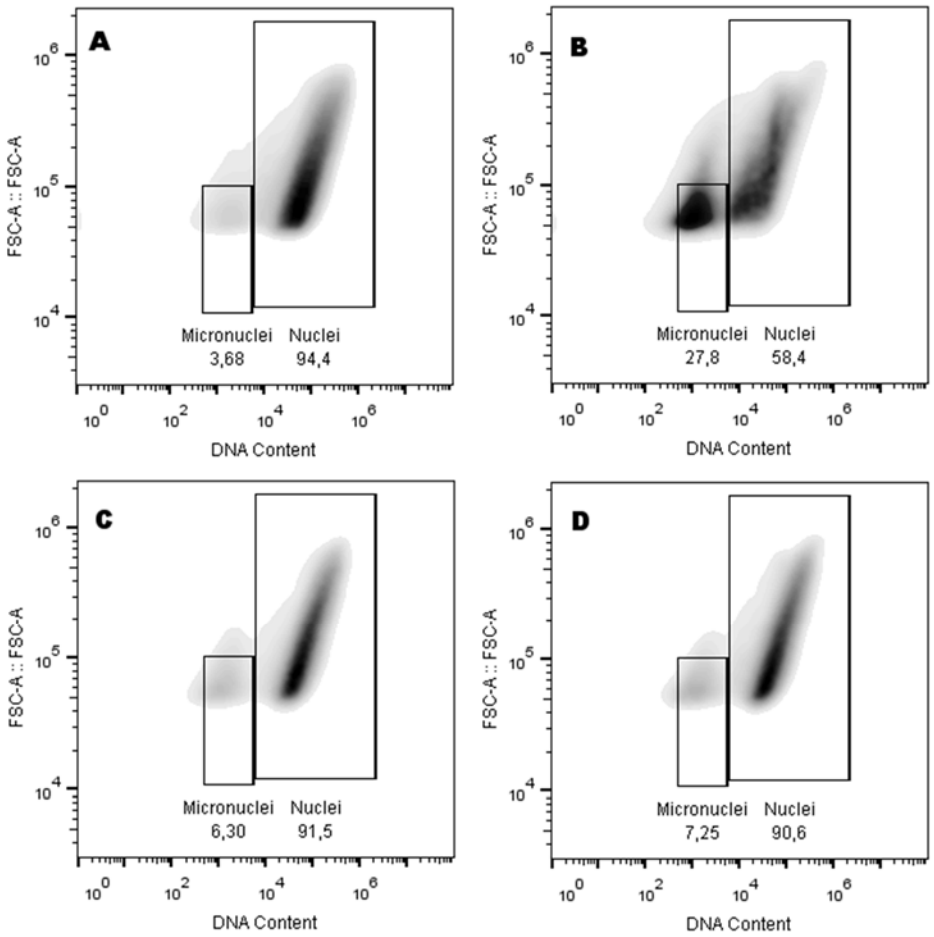

Figure 2: Examples of results from experimental groups. (A) NaCl 0,9%; (B) Ciclophosphamide, (C) MMS, (D) Irradiated diet

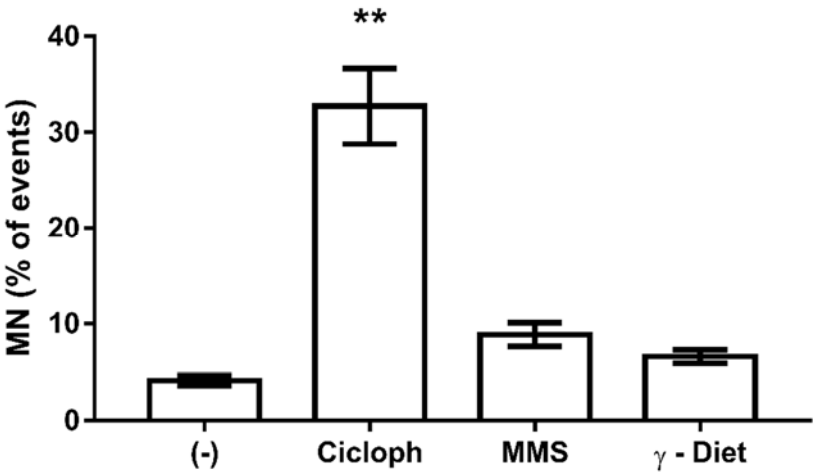

Figure 3: Percentage of MN (% of total events) of liver cells of rats treated with NaCl (-), Ciclophosphamide, MMS or irradiated diet (γ-diet). Bars: SEM. (**): p<0,01 (difference from negative control)

3. CONCLUSIONS

Although MMS did not induce micronuclei statistically different percentages from that found in the controls, cyclophosphamide induced damage, which leads to validation of the assay and to verify that there was no genotoxic damage in hepatic cells from animals fed for 8 months with irradiated diet, supposedly containing 2-ACBs. Further experiments (histopathology, visceral fat, hematology) will corroborate results.

REFERENCES

In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity Steven M. Bryce, Jeffrey C. Bemis, Svetlana L. Avlasevich, Stephen D. Dertinger Mutat Res. Author manuscript; available in PMC 2007 Aug 22. Published in final edited form as: Mutat Res. 2007 Jun 15; 630(1-2): 78–91. Published online 2007 Mar 19. doi: 10.1016/j.mrgentox.2007.03.002