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Evaluation of the Immunological Cellular Response of *Cebus apella* Exposed to the Carcinogen *N*-Methyl-*N*-nitrosourea and Treated with CANOVA®

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Abstract. *The immune response modifier Canova® is a homeopathic remedy indicated for patients with depressed immune system, since this drug appears to increase adaptive immunity and induce an immune response against multiple and severe pathological conditions, including cancer. We evaluated the pattern of immune cellular response in non-human primates of the species Cebus apella exposed to N-methyl-N-nitrosourea (MNU) with and without Canova®. Twelve animals were divided into four groups, with three animals each: negative control and three experimental groups, MNU-alone (35 days); MNU (35 days)-plus-Canova® (3 days) and Canova®-alone (3 days). The animals received MNU orally and Canova® by three intravenous injections. Evaluation of the cellular immune response was performed by immunophenotyping of T-lymphocytes ($CD4^+$, $CD8^+$), B-lymphocytes and natural killer cells. Analysis was also performed of the cell cycle. Our results suggest an increase of T-lymphocytes ($CD4^+CD3^+$) only in the Canova® group, while in the MNU-plus-Canova® group only B-lymphocytes increased.*

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In recent years, several compounds have been tested as potential new adjuvant therapies to protect healthy tissues from the toxic effects of radiation, anticancer drugs, or carcinogenic substances in the environment (1). These new therapies can serve as chemoprotectants, protecting against the toxicity of antineoplastic agents without compromising antitumour efficacy, or as immunomodulators, substances which modify the immunity of an individual to favor a specific immune response. This can provide improvement in quality of life during treatment with anti-neoplastic agents (2, 3).

Canova® (product code NDC 58088-001; Canova do Brazil®, listed and regulated by the Food and Drug Administration, USA), being a homeopathic active immune response modifier, is primarily indicated for patients whose immune system is depressed (4, 5). Canova® treatment appears to increase an individual's ability to trigger a specific immune response against various pathological conditions, including cancer (3, 6). This increase of innate immunity is due to its involvement in the proliferation and differentiation of hematopoietic cells and induction of mononuclear differentiation of bone marrow cells (7).

N-Methyl-*N*-nitrosourea (MNU) is a alkylating agent with powerful carcinogenic ability to cause mutations, chromosomal aberrations and DNA methylation, which can induce an imbalance in the defense system of the cell and thus stop mechanisms related to cellular metabolism. It has been widely used in the induction of experimental tumors (8, 9).

The oral administration of MNU is characterized by the induction of squamous cell carcinoma of the gastrointestinal surface in monkeys (mouth, pharynx, larynx and stomach), as well as chronic inflammation generally observed in the

esophagus causing increased cell proliferation (10, 11). When used at high concentrations, MNU first targets the lymphoid hematopoietic system (12). Thus, tumorigenesis induced by MNU is an interesting model for studying the immune response.

Due to the close phylogenetic relationship to humans of non-human primates and their greater similarities with regard to anatomy, physiology, biochemistry, and organ systems compared to rodents, they also provide a useful model for studying carcinogens and for the development and validation of new therapies for various diseases that affect humans (10, 13).

The goal of the present work was to assess the pattern of hematopoietic cell response in the primate species *Cebus apella* when exposed to carcinogenic MNU and subjected to treatment with Canova® through the analysis of immune parameters and the cell cycle.

Materials and Methods

We used 12 adult animals of the species *Cebus apella* in captivity at the National Primate Center maintained under the conditions of the same patterns, which are: primates housed in individual sections within aluminum cages with dimensions of 80×90×80 cm, sheds, subject to natural photoperiod and fed daily with fruit, vegetables, superworms (*Zophobas morio at the larval stage*), pelleted food (FOXY Junior Supreme, 28% crude protein; PROVIMI, Pinhais, PR, Brazil) and water ad libitum. For identification, animals had a microchip implanted in the dorsal interscapular region. The animals were weighed before and after every blood collection throughout the experimental period. According to a basic veterinary examination, all animals were considered healthy at the time of first blood sampling. This study was approved by the Ethics Committee of Federal University of Pará (PARECER MED002-10).

The animals participating in the study group consisted of a total of 12 adult animals with about eight years old, male, average weight of 3.725 kg. The animals were divided into four groups, with three animals each: negative control (received saline solution instead of MNU/Canova®) and three experimental groups, MNU alone for 35 days; MNU for 35 days plus Canova® for three days; and Canova® alone for three days. Each animal received daily a solution of 16 mg/kg MNU (Sigma-Aldrich Brazil LTDA, São Paulo, SP, Brazil) according to the protocol used by Thorgeirsson *et al*. (11) and Takaya orallyma *et al*. (14). Canova® (Canova do brazil®, Curitiba, PR, Brazil) treatment was performed by three intravenous injections at the dose of 1.67 ml/kg for three consecutive days, determined according to the study of Borges da Costa *et al*. (15) in *C. apella* which evaluated different applications of Canova®. Blood samples were obtained at seven-day intervals after treatment with MNU, and 24 and 96 hours and 15 days after treatment with Canova®, for evaluating the cellular immune response by immunophenotyping [$CD4^+$, $CD8^+$, $CD3^+$, B-lymphocytes and natural killer (NK) cells, with the acquisition of 10000 events, according to the protocol used by Brito Junior *et al*. (16).

Figure 1 shows the experimental design utilized, with the collection days (arrow) and treatment with MNU/Canova® according to the study groups.

The cell cycle was examined by lymphocyte culture. The lymphocyte culture was obtained from whole peripheral blood collected with heparin from all animals, cultivated in RPMI complete medium (Cultilab, Campinas, PR, Brazil) incubated at 37˚C for 72 hours. Cells from the culture of lymphocytes were analyzed using the Test™ Cycle Kit PLUS DNA Reagent (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) according to the manufacturer's protocol, and subsequent processing of the samples by FACS Calibur flow cytometry, using Cell Quest Pro system. The data were subjected to statistical analysis by BioEstat® 5.0 software (Civil Society Mamirauá, Belém, PA, Brazil), with α=0.05 level of significance (5%) through one-way analysis of variance (ANOVA) and differences between samples were determined by Bonferroni multiple comparison test.

Results

The results of the immunophenotypic profile of lymphocytes from *C. apella* treated with MNU with/without Canova® are presented in Table I. Means and standard deviations of the absolute values of B-lymphocytes ($CD19^+$), total T-lymphocytes ($CD3^+$), T-helper lymphocytes ($CD4^+CD3^+$), cytotoxic T-lymphocytes ($CD8^+CD3^+$) and NK cells ($CD3^+/CD16^+/CD56^+$) are presented.

In the experimental groups, treated with MNU for 35 days plus Canova® for three days, we observed an increase in total T-lymphocytes ($CD3^+$). The same groups showed a pattern of increase in T-helper lymphocytes ($CD4^+CD3^+$). In the groups treated with Canova®-alone, the total B-lymphocytes ($CD19^+$) increased, but this cell type is also increased in the groups treated with MNU for 35 days plus Canova® for three days.

The cell-cycle kinetics of *C. apella* lymphocytes was analyzed by flow cytometry to determine possible changes induced by the carcinogen MNU, and to evaluate possible protective effects of Canova®. The distribution of cells in different cell-cycle phases was based on relative differences in DNA content between cells analyzed during the pre-replicative (G_0/G_1), replicative (S phase) and post-replicative (G_2+M) phases. The only significant difference found in the distribution of cells in the cell cycle was in the group exposed to MNU alone for 35 days compared to the negative control group ($p<0.05$), in which there were significantly fewer cells in the G_0/G_1 and G_2+M phases.

In cell-cycle analysis, in the group that received MNU for 35 days, the percentage of G_0/G_1 cells was significantly lower, when compared to groups receiving MNU for 35 days plus Canova® for three days and Canova® alone. In relation to the S phase of the cell cycle, the percentage was significantly higher when compared to the group that received Canova® only, and mean values of G_2+M phase cells were significantly lower compared with the group that received Canova® only. These results are presented in Table II.

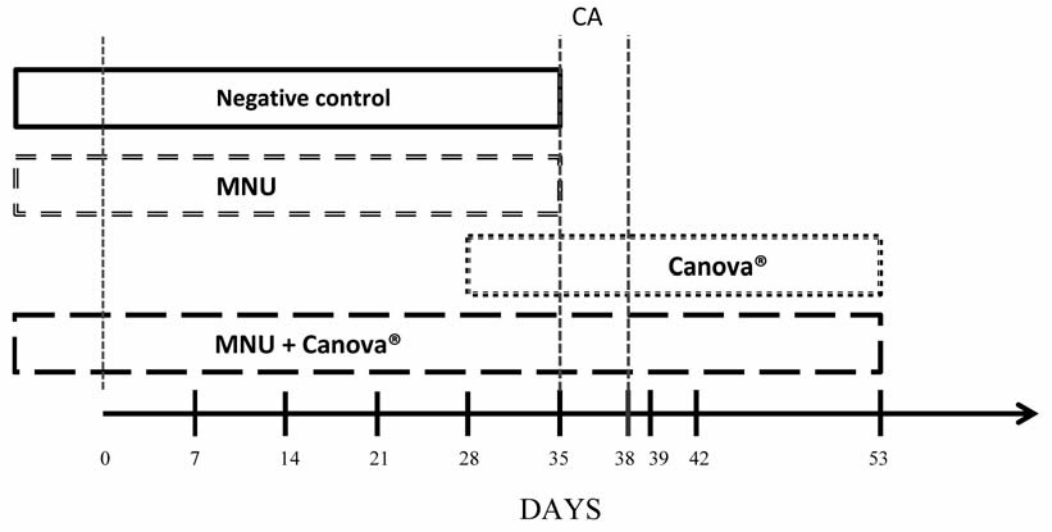

Figure 1. *Schematic of the experimental design. MNU: N-Methyl-N-nitrosourea and CA: Canova®.*

Table I. *Phenotypic characterization by flow cytometry of T-and B-lymphocytes and natural killer (NK) cells in blood of Cebus apella after treatment with N-methyl-N-nitrosourea (MNU) for 35 days, Canova® (CA) alone for 3 days, MNU for 35 days plus CA for 3 days, and in the negative control group (NC; received saline solution).Data are expressed as mean±SD of the absolute acquisition of 10000 events from 50 µl of blood.*

Immunological markers	NC Mean±SD	MNU Mean±SD	MNU+CA Mean±SD	CA Mean±SD
T-Helper lymphocytes ($CD4^+CD3^+$)	1357±147.75	1344 ±383.43	2542±535.29*†	1634±691.80
T-Lymphocytes ($CD3^+$)	1969±85.15	2211±445.22	3610±568.79*†	2527±662.58
Cytotoxic T-lymphocytes ($CD8^+CD3^+$)	611±86.33	1166±454.60	1068±398.49	991±293.58
NK cells	6±8.19	3±3.49	3±3.36	11±14.31
B-Lymphocytes ($CD19^+$)	1224±265.20	1721±481.32	2652±386.94*†	2142±574.90*

*Significantly different from NC at $p<0.05$; †significantly different from MNU-alone and from CA-alone at $p<0.05$.

Discussion

Animals treated with MNU alone for 35 days did not show any change in the number of T-cells ($CD3^+$, $CD4^+$ and $CD8^+$), B-lymphocytes and NK cells. Our results corroborate those of Spinardi-Barbisan *et al*. (17), who demonstrated that MNU did not change the number of immune cell after four weeks of exposure. MNU is a carcinogen with a wide range of side-effects due to its toxicity, however, it has been shown that this toxicity is temporary and that the bone marrow is able to restore immune cells to basal levels after three weeks post-MNU treatment (18-20).

On the other hand, animals treated with Canova® showed an increase in B-lymphocytes ($CD19^+$) and T-helper cells ($CD4^+CD3^+$) and total T-cells ($CD3^+$). Our results are in accordance with Sato *et al*. (21) where mice bearing sarcoma

Table II. *Characterization of the cell cycle distribution by flow cytometry of cultures of lymphocytes from Cebus apella after treatment with N-methyl-N-nitrosourea (MNU) for 35 days, Canova® (CA) alone for 3 days, MNU for 35 days plus CA for 3 days, and in the negative control group (NC;received saline solution). Data are mean±SD, based on the percentage obtained in the acquisition of 5000 events from 100 µl of sample culture of lymphocytes.*

Group	DNA content (%)		
	G_0-G_1	S	G_2-M
NC	63.96±5.22	22.24±4.32	7.85±2.13
MNU	53.16±14.83*	25.40±13.88†	4.27±3.83†
MNU+CA	65.04±5.31	17.21±4.79	4.64±2.49
CA	63.05±4.70	21.02±4.63	6.64±1.59

*Significantly different from MNU+CA and CA alone at $p<0.05$; †significantly different from CA alone at $p<0.05$.

180 (S-180) treated with Canova® showed an increase in B-lymphocytes and NK cells, especially improving T-helper cell ($CD4^+$) response. In our study, we did not find an improvement in NK cells, probably due to the differences in the adaptation of the immune system in *C. apella* compared to mice. It is also known that Canova® stimulates progenitor cells in the bone marrow, leading to activation and differentiation of monocytic cell lineage (CD11b), stromal cells (7), and mononuclear cells (6).

Animals treated with MNU-alone exhibited a blockage of cells in the S-phase, with a reduction of G_0/G_1 and G_2/M phases. These data corroborate these of Kirkhus *et al*. (22), where mouse epidermal cells treated with MNU exhibited a delay in the cell cycle, and accumulation of the cells in the S and G_2 phases. Hebels *et al*. (23) also showed that Caco-2 cells (colon cancer) treated with MNU accumulated in the S-phase, triggering cells to undergo apoptosis (23).

Animals treated with MNU-plus-Canova® did not show significant changes in the cell cycle distribution when compared to those treated with Canova® alone, suggesting that Canova® treatment overcame the cell cycle blockage induced by MNU (Table II), restoring cells to the basal distribution (negative control). To the best of our knowledge, this is the first time that the cell cycle kinetics and protection by Canova® has been described in an *in vivo* study, especially in a non-human primate model. These results corroborate previous studies where Canova® treatment protected cells from death, promoting cell proliferation and differentiation, directly or indirectly (6, 24-28).

Taken together, the results suggest that the immune response modifier Canova® is capable of supporting the immune system by increasing the number of T- ($CD4^+$) and B-lymphocytes, protecting cells from cell-cycle damage, and thus might be useful as an adjuvant in cancer therapy by reducing side-effects of chemotherapy.

Conflicts of Interest

All Authors declare that they have no conflicts of interest.

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