

Research Article**Evaluation of the Biochemical, Hematological and Genotoxic Parameters of *Cebus Apella* Treated with N-Methyl-Nitrosourea (NMU) Followed by Treatment with a Complex Homeopathic Compound (Canova)**

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Abstract: Canova is a homeopathic compound that presents antimutagenic and anticancer properties. N-methyl-nitrosourea is a carcinogenic agent that causes alterations on DNA. This study was conducted to evaluate the antimutagenic effects of Canova on *Cebus apella* non-human primates treated with N-methyl-nitrosourea. Six animals were randomly assigned in two groups, one composed by two animals, who received only N-methyl-nitrosourea (NMU group) and another one consisting on four animals treated with N-methyl-nitrosourea followed by Canova treatment (CA group). Body weight, biochemical and hematological analysis were performed. Micronuclei test and Comet assay were carried out to evaluate genotoxicity. N-methyl-nitrosourea treatment led to an overall reduction in the hematologic cell count and the administration of Canova limited the adverse effects in the hematologic system on CA group. Canova also reduced micronuclei frequency and the DNA damage index. It suggests that Canova may have a role as an immune response modulator by inducing leukocyte proliferation.

Keywords: Canova; N-methyl-N-nitrosourea; carcinogen exposure; non-human primates; *Cebus apaella*

Introduction

Canova (CA) is a complex homeopathic immune response modulator indicated for patients with depressed immune system. CA activates

macrophages both *in vivo* and *in vitro* and indirectly induces lymphocyte proliferation^[1]. Experiments *in vivo* and *in vitro* point out that treatment with CA can cause tumor size reduction and regression

in animals, and can also recover cells viability after they were damaged^[2].

NMU was successfully used as an inductor of gastric carcinogenesis in *Cebus apella* and the treatment with CA was able to restore several hematologic measurements in those animals, suggesting that CA can be used during/after chemotherapy to increase the tolerability and duration of anticancer treatments^[3].

Thus, the aim of the present study was to evaluate possible protective effects mediated by CA against the genotoxicity caused by oral MNU ingestion in non-human primates of the specie *Cebus apella*.

Materials and Methods

Canova: CA was purchased from suppliers authorized by the Brazilian Health Ministry. The final product contains *Aconitum napellus* dH20, *Apis mellifica* dH19, *Arsenicum album* dH17, *Asa foetida* dH20, *Baryta carbonica* dH20, *Bryonia alba* dH14, *Calcarea carbonica* dH20, *Conium maculatum* dH16, *Ipecacuanha* dH13, *Lachesis muta* dH18, *Lycopodium clavatum* dH20, *Pulsatilla nigricans* dH13, *Rhus toxicodendrum* dH17, *Ricinus communis* dH14, *Silicea* dH18, *Thuya occidentalis* dH16, *Veratrum album* dH20 and less than 1% ethanol in distilled water. Experiments were performed with commercial CA donated by 'Canova do Brasil' (www.canovado brasil.com.br). Our research group has previously demonstrated that CA treatment does not induce cytotoxic or genotoxic effects at chromosomal level^[4].

Animals: Six male adults of *Cebus apella* (6–8 years old, ranging 2.8–3.7 kg) were included in this study. Animals were identified with microchips and were individually housed in Centro Nacional de Primatas, Pará State, Brazil. The animals were fed with a healthy balanced diet not enriched with sodium chloride. Water was restricted during MNU treatment and given *ad libitum* after MNU treatment. This study received the approval of the Ethics Committee of Universidade Federal do Pará (MED002/2007). According to basic veterinary examination, all animals were considered healthy at the time of the first blood sampling.

Animal Evaluation: During the treatment period, body weight was determined and peripheral blood was collected daily. Chemistry analysis included glucose, urea nitrogen, creatinine, albumin, total bilirubin, cholesterol, triglyceride, alanine aminotransferase and

inorganic phosphorus. The hematologic parameters analyzed included red blood cell count, platelet count, white blood cell (WBC) and differential (segmented neutrophil, lymphocyte, monocyte, eosinophil and basophile) counts. Methods and reference values for adult animals were previously described^[5]. For this study, we used the serum values of days 0 (before the drug treatment), 30, 60, 90, 240, 242, 244, 246 and 270.

All animals were carefully monitored to avoid pain signals, discomfort, stress or infections before, during and after drug injections. Allergic and toxic chemo-related reactions were also evaluated, including the analysis of chromosomal aberration and DNA damage due to MNU effects.

Experimental Design: The animals were randomly assigned in two groups, who didn't differ on baseline measurements. The first group was composed by two animals receiving MNU (control group). The second group consisted on four animals treated with MNU followed by CA (CA group). The CA group received three injections of 1.67 µL/g commercial CA in days 240, 242 and 244, after the end of MNU treatment. Animals were weighed daily and the doses calculated at that time. CA solution was vigorously shaken (succussion) immediately before treatment. CA was injected by slow infusion in the right femoral vein of *C. apella* in a single dose. Animals received daily oral doses of MNU (N1517 Sigma-Aldrich, USA) for 240 days at a dosage of 16 mg/kg body weight. Daily, animals received drinking water containing MNU in light-shielded bottles. All animals were monitored during a total of 270 days.

Cell Culture: Peripheral blood samples were collected from all *C. apella* on days 0 (before treatment), 30, 60, 90, 240, 242, 244, 246 and 270. Samples obtained in the days 242, 244, and 246 were collected before CA administration. 0.5 µL of whole blood were seeded in 5 mL of RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 20% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 4% phytohemagglutinin A (Gibco-Invitrogen, Carlsberg, CA), 0.05 mg/mL gentamicin sulfate (Cultilab, Campinas, SP, Brazil), and 0.002 mg/mL amphotericin B. Cells were cultured at 37 °C in an incubator containing 5% CO₂.

Micronucleus Test (MN): Forty-four hours after the culture started, 25 µL of Cytochalasin B (Sigma Chemical Co., St. Louis) (3 µg/mL)

were added to each culture in order to obtain binucleated cells and re-incubated for another 28 h. Seventy-two hours after the beginning of incubation, cells were harvested, centrifuged (5 min at 800 rpm) and quickly treated with a hypotonic solution (KCl 0.075 M). Afterward, cells were washed once with 5 mL of a cold methanol:acetic acid (5:1) (V/V) fixative and washed again with 5 mL of a cold methanol:acetic acid solution (3:1) (V/V). The cell suspension was dropped onto slides and stained in a solution of 5% Giemsa dye (Sigma Chemical Co., St. Louis) in phosphate buffer (pH 6.8) for 5 min. Micronuclei (MN) were scored in 1,000 binucleated cells using the criteria described elsewhere^[6].

Cell Viability and Alkaline Comet Assay: Before comet assay, the cell viability was analyzed by trypan blue exclusion. Cells of whole blood were stained with 0.25% (W/V) trypan blue (T8154 Sigma-Aldrich, USA). The unstained (viable) cells were counted under light microscopy. 100 cells were counted to determine the percentage of viable cells excluding trypan blue. All samples presented cell viability greater than 75% and were used for comet assay.

Alkaline comet assay was performed as described by Singh *et al.*^[7], with minor modifications.

Data Analysis: Friedman test was applied at 0, 30, 60 and 90 day evaluations to the micronucleus, DNA DI, serum biochemistry and hematologic results. During this period, Mann-Whitney was performed to compare the mean of MN, DNA DI and serum biochemistry and hematologic parameters between CA and control groups. In all analyses, *P* values less than 0.05 were considered significant. Due to the reduction of animals in the NMU group caused by death, the statistical data analysis concerning days 240–270 was not possible and results are presented in a descriptive format. The correlation between lymphocyte count and DNA DI was analyzed by Pearson's test and between MN frequency and lymphocyte count or DNA DI by Spearman's test.

Results

The present study began with six animals. However, three animals died due to MNU intoxication on days 110, 134, and 140. These animals showed the typical symptoms of intoxication: mydriasis, confusion, sleepiness, giddiness, loss of balance, tremor, hyperthermia, low food consumption, nonspecific gastrointestinal

symptoms (diarrhea and vomiting), urinary retention, cutaneous eruptions and caustic and ulcerative oral lesions. They also presented renal, hepatic and respiratory failure, hypokalemia, chronic cholecystitis, steatosis, elevation of alanine aminotransferase, bilirubin and creatine levels. Thus, for the second phase of the present study, we only had a restricted number of animals to evaluate CA effects and only a descriptive analysis was possible between days 240–270. No tumor was observed in the three surviving animals during the 4 months after this study.

Table 1 shows weight, biochemical and hematologic measurements, MN frequency, and DNA DI in *C. apella*. Animal weight decreased significantly between days 0–90 ($P=0.0033$). Concerning biochemistry evaluations, a significant increase in urea nitrogen was observed ($P=0.0004$), inorganic phosphorus ($P=0.0004$), triglycerides ($P=0.0007$), alanine aminotransferase ($P=0.0008$), total bilirubin ($P=0.0008$) and creatinine ($P=0.0006$) levels during MNU treatments. Albumin ($P=0.0009$) and cholesterol ($P=0.0056$) decreased significantly with MNU treatments (Table 2). According to the normal levels of *C. apella* serum chemistry values^[5], all animals presented an abnormally elevated inorganic phosphorus after day 90 (dead animals) or day 240. All animals also presented an abnormally elevated level of alanine aminotransferase. A slightly increase of total bilirubin level was observed in the dead animals at day 90. The only surviving animal of the control group presented an abnormal increase of urea. Before and during the MNU treatment, no biochemical parameter was significantly different between the CA and control groups.

Concerning hematologic analysis, we observed a significant reduction in erythrocyte ($P=0.0004$), leukocyte ($P=0.0004$), lymphocyte ($P=0.0007$) and neutrophil ($P=0.0004$) counts during treatment with MNU (Table 2). Before treatment and during MNU treatment, no hematologic value was significantly different between the CA and the control groups. However, according to the normal levels of *C. apella* hematologic values^[5], only the surviving animal of the control group presented an abnormally reduced neutrophil count. Concerning lymphocyte counts, only one animal of the CA group present an abnormal count (day 242) and the surviving animal of the control group presented a reduced lymphocyte count between days 240–270. Although all animals presented abnormal values of erythrocytes due to

MNU treatment, we observed that the erythrocyte count increased with CA treatment. Moreover, we also observed that CA contributes

to a fast increase in leukocyte count, including the lymphocytes and neutrophils.

Table 1. Weight, biochemical and hematologic measurements, micronuclei frequency, and DNA damage index in *C. apella*.

Measurement	Group	Days (median ± interquartile range)								
		Baseline	30	60	90	240 ^{a,b}	242 ^{a,b}	244 ^{a,b}	246 ^{a,b}	270 ^{a,b}
Weight (kg)	CA	3.00 ± 0.25	2.80 ± 0.25	2.75 ± 0.48	2.35 ± 0.28	2.50 ± 0.40	2.60 ± 0.40	2.80 ± 0.40	3.00 ± 0.40	2.95 ± 0.45
	CN	3.55 ± 0.15	3.25 ± 0.15	2.95 ± 0.25	2.6 ± 0.10	2.7	2.5	2.7	2.8	2.8
Inorganic phosphorus (mg/dL)	CA	2.32 ± 0.35	3.26 ± 0.33	4.52 ± 0.78	4.98 ± 0.93	6.25 ± 0.23	6.26 ± 0.32	6.25 ± 0.19	5.96 ± 0.14	4.39 ± 0.19
	CN	2.50 ± 0.16	2.67 ± 0.14	3.83 ± 0.84	5.67 ± 1.06	5.05	5.1	5.8	5.82	6.73
Urea nitrogen (mg/dL)	CA	13.90 ± 0.92	16.52 ± 1.02	19.36 ± 2.19	21.98 ± 5.08	23.97 ± 0.23	23.11 ± 0.53	23.45 ± 0.47	22.17 ± 0.45	18.54 ± 1.33
	CN	13.75 ± 0.64	15.47 ± 0.96	16.77 ± 0.95	20.24 ± 2.15	25.64	25.8	25.67	25.86	28
Alanine aminotransferase (U/L)	CA	41.50 ± 2.25	49.00 ± 11.75	57.00 ± 14.50	57.00 ± 11.50	60.00 ± 1.00	60.00 ± 1.00	58.50 ± 1.50	58.00 ± 0.00	47.00 ± 2.00
	CN	36.00 ± 1.00	40.00 ± 1.00	43.00 ± 1.00	50.00 ± 8.00	56	57	56	51	46
Total bilirubin (mg/dL)	CA	0.53 ± 0.012	0.57 ± 0.05	0.59 ± 0.05	0.69 ± 0.19	0.65 ± 0.06	0.65 ± 0.06	0.64 ± 0.05	0.64 ± 0.06	0.57 ± 0.01
	CN	0.53 ± 0.01	0.56 ± 0.01	0.61 ± 0.04	0.65 ± 0.09	0.69	0.69	0.68	0.69	0.58
Triglycerides (mg/dL)	CA	97.62 ± 6.45	101.06 ± 11.10	105.39 ± 11.93	108.95 ± 10.89	112.73 ± 3.24	114.28 ± 3.73	114.80 ± 2.04	112.12 ± 0.51	109.18 ± 0.93
	CN	106.55 ± 1.88	106.71 ± 2.50	111.49 ± 3.10	114.81 ± 3.99	125.74	129.5	128.93	126.26	121.1
Glucose (mg/dL)	CA	76.22 ± 1.12	76.45 ± 2.45	75.80 ± 4.66	75.19 ± 3.86	76.74 ± 4.03	75.07 ± 4.62	76.40 ± 3.62	76.73 ± 3.28	75.66 ± 1.66
	CN	77.56 ± 0.44	77.77 ± 0.57	74.03 ± 2.79	71.10 ± 2.35	70.27	68.4	69.49	70.82	73.66
Albumin (%)	CA	64.09 ± 2.17	63.03 ± 1.70	61.41 ± 1.24	60.54 ± 1.86	61.20 ± 1.68	60.98 ± 1.81	60.75 ± 1.40	60.72 ± 1.44	60.52 ± 0.48
	CN	61.84 ± 2.63	60.93 ± 2.29	60.71 ± 2.48	60.27 ± 2.93	55.75	55.87	55.32	55.93	56.73
Cholesterol (mg/dL)	CA	157.59 ± 18.00	156.56 ± 17.78	158.40 ± 20.26	153.58 ± 16.61	148.20 ± 10.03	147.46 ± 9.24	148.24 ± 7.60	148.91 ± 9.01	150.89 ± 7.81
	CN	165.67 ± 2.02	161.29 ± 1.29	158.39 ± 0.66	155.60 ± 1.69	152.76	152.13	152.3	150.21	151.36
Creatinine (mg/dL)	CA	0.61 ± 0.04	0.73 ± 0.12	0.78 ± 0.06	0.82 ± 0.05	0.86 ± 0.06	0.86 ± 0.03	0.85 ± 0.02	0.94 ± 0.02	0.83 ± 0.06
	CN	0.70 ± 0.06	0.74 ± 0.06	0.79 ± 0.07	0.86 ± 0.08	0.89	0.9	0.93	0.97	0.84
Leukocytes (10 ³ /μL)	CA	7.97 ± 0.44	6.75 ± 1.56	5.76 ± 1.23	4.88 ± 0.59	4.58 ± 0.69	4.63 ± 0.24	5.95 ± 0.11	7.19 ± 0.47	7.56 ± 0.66
	CN	8.18 ± 0.26	6.73 ± 0.50	6.01 ± 0.70	5.12 ± 0.23	4.56	4.57	4.22	4.19	3.8
Lymphocytes (10 ³ /μL)	CA	35.43 ± 0.91	32.60 ± 2.53	32.41 ± 3.20	31.02 ± 3.33	20.17 ± 1.61	21.22 ± 0.34	33.51 ± 1.84	37.00 ± 0.58	37.51 ± 0.51
	CN	35.65 ± 0.68	30.30 ± 0.17	28.78 ± 0.26	27.07 ± 0.36	19.9	17.88	17.22	16.96	15.94
Neutrophil (10 ³ /μL)	CA	60.62 ± 1.63	57.12 ± 2.77	51.43 ± 3.10	48.34 ± 7.79	48.62 ± 0.61	50.99 ± 0.48	53.18 ± 0.59	54.75 ± 0.80	58.11 ± 1.29
	CN	59.73 ± 0.5	55.60 ± 0.75	53.26 ± 0.54	50.78 ± 0.71	39.94	36.66	35	34.32	30
Erythrocytes (10 ⁶ /mm ³)	CA	6.34 ± 0.24	4.97 ± 0.27	3.68 ± 1.42	3.22 ± 1.91	2.47 ± 0.32	3.23 ± 0.19	3.81 ± 0.24	4.54 ± 0.42	5.71 ± 0.20
	CN	5.96 ± 0.07	4.66 ± 0.21	44.12 ± 0.09	4.01 ± 0.14	1.91	2.13	2.04	1.96	1.92
Eosinophils (10 ³ /μL)	CA	0.02 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	-	-	-	-	0.02 ± 0.01	0.02 ± 0.01
	CN	0.01 ± 0.00	-	-	-	-	-	-	-	-
Micronuclei frequency	CA	2.00 ± 0.25	20.50 ± 2.50	33.50 ± 4.75	39.50 ± 9.25	155.00 ± 12.00	127.00 ± 7.00	115.00 ± 10.00	102.50 ± 3.50	38.50 ± 2.50
	CN	2.5 ± 0.50	28.50 ± 6.50	35.00 ± 4.00	40.00 ± 3.00	138	163	168	175	189
DNA damage index	CA	33.00 ± 4.25	66.25 ± 3.50	95.25 ± 3.63	107.75 ± 6.50	166.00 ± 19.50	157.25 ± 20.25	151.25 ± 21.25	137.00 ± 22.00	81.50 ± 20.00
	CN	33.25 ± 4.75	66.75 ± 9.25	96.25 ± 7.25	116.50 ± 14.00	138.5	157	168.5	178.5	190

CA: *Canova* group – animals treated with MNU for 240 continuous days and treated with *Canova* in days 240, 242 and 244 after the end of MNU treatment. CN: negative control group – animals treated with MNU for 240 continuous days. ^a Analyses based in two animals for *Canova* group. ^b Analyses based in one animal for negative control group.

Table 2. Weight, biochemical and hematologic measurements, micronuclei frequency, and DNA damage index in six *C. apella* during 90 days of MNU treatment.

Measurement	Days (median ± interquartile range)			
	Baseline	30	60	90
Weight (kg)	3.15 ± 0.40	2.95 ± 0.33	2.90 ± 0.50	2.45 ± 0.33*
Inorganic phosphorus (mg/dL)	2.38 ± 0.35	2.98 ± 0.52*	4.45 ± 1.07*	4.98 ± 1.37* [§]
Urea nitrogen (mg/dL)	13.90 ± 1.07	16.39 ± 0.80*	18.30 ± 1.96*	20.98 ± 4.38* [§]
Alanine aminotransferase (U/L)	39.50 ± 4.50	45.00 ± 9.00	50.00 ± 14.75*	56.00 ± 7.75*
Total bilirubin (mg/dL)	0.53 ± 0.01	0.56 ± 0.03	0.59 ± 0.07*	0.67 ± 0.19*
Triglycerides (mg/dL)	102.05 ± 9.66	104.94 ± 9.86	107.92 ± 8.67	110.41 ± 8.38* [§]
Glucose (mg/dL)	76.68 ± 1.57	77.34 ± 1.96	75.25 ± 4.11	73.64 ± 2.80
Albumin (%)	63.97 ± 2.80	62.97 ± 1.88	61.41 ± 2.43*	60.54 ± 2.71*
Cholesterol (mg/dL)	164.11 ± 13.04	161.29 ± 11.35	158.39 ± 15.04	155.60 ± 12.82* [§]
Creatinine (mg/dL)	0.63 ± 0.10	0.73 ± 0.11	0.78 ± 0.09	0.82 ± 0.05*
Leukocytes (10 ³ /μL)	7.97 ± 0.41	6.75 ± 0.99*	5.76 ± 1.28*	4.89 ± 0.36* [§]
Lymphocytes (10 ³ /μL)	35.53 ± 0.99	31.49 ± 2.36	30.24 ± 3.95	28.64 ± 4.29*
Neutrophil (10 ³ /μL)	60.12 ± 1.56	56.29 ± 2.11*	52.90 ± 2.62*	50.78 ± 5.48* [§]
Erythrocytes (10 ⁶ /mm ³)	6.17 ± 0.39	4.90 ± 0.19	4.12 ± 0.91*	3.96 ± 1.36* [§]
Eosinophils (10 ³ /μL)	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.00 ± 0.00
Micronuclei frequency	2.00 ± 0.00	21.50 ± 3.25*	33.50 ± 6.25*	39.50 ± 5.75* [§]
DNA damage index	33.00 ± 4.75	66.25 ± 7.50*	95.25 ± 7.88*	107.75 ± 16.50* [§]

* $P < 0.05$

We also observed a significant increase in MN frequency ($P=0.0004$) and DNA DI ($P=0.0004$) with MNU treatment (Table 2). Before treatment and during MNU treatment, no significant difference between the CA and control groups was observed concerning MN frequency and DNA DI. However, we observed that the surviving animal of the control group presented an elevated frequency of MN and DNA DI after MNU treatment and, on the other hand, the animals of the CA group presented a decline in these parameters due to the homeopathic medicine.

Fig. 1 shows the inorganic phosphorus, urea nitrogen, alanine aminotransferase and total bilirubin levels, leukocyte, lymphocyte, neutrophil and erythrocyte counts, MN frequency and DNA DI observed in the CA and control groups during a 270 day period.

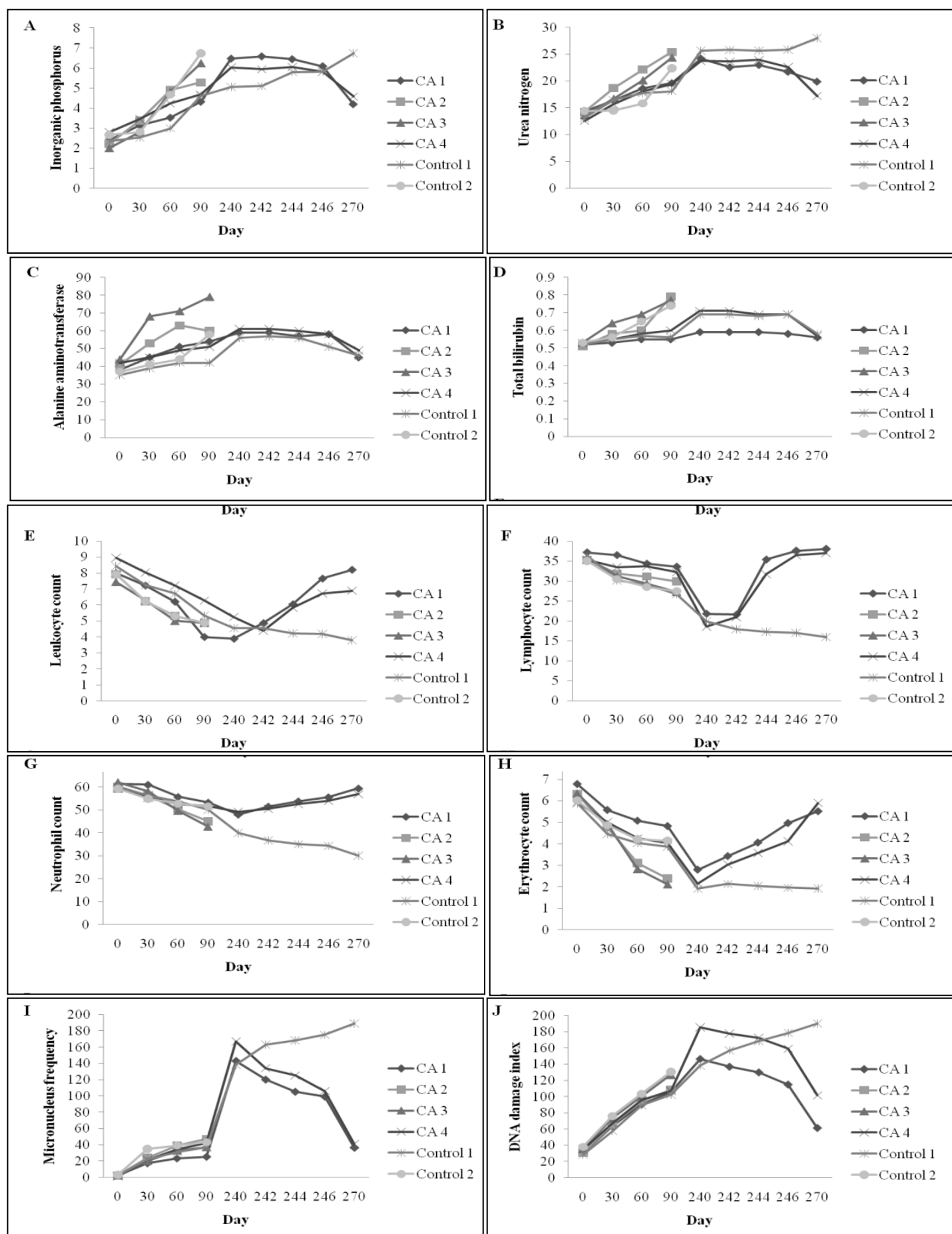


Fig. 1: Individual values of 4 animals treated with *Canova* (CA) and 2 animals of control group during a 270 day period. A: inorganic phosphorus level (mg/dL); B: urea nitrogen level (mg/dL); C: alanine aminotrasferase level (U/L); D: total bilirubin (mg/dL); E: leukocyte count ($10^3/\mu\text{L}$); F: lymphocyte count; G: neutrophil count; H: erythrocyte count; I: micronucleus frequency; J: DNA damage index.

Discussion

Abud *et al.*^[8] reported that CA-activated macrophages induced the production of lymphocytes and erythrocytes. Our group also previously observed that CA induces macrophage activation and indirectly leads to lymphocyte proliferation *in vitro*^[11]. CA activated macrophages present an increase in the intracellular activity of the NAD(P)H oxidase and Nitric Oxide Sintetase (NOS) enzymes, therefore stimulating the production of reactive oxygen species (ROS) and nitric oxide, which might induce gene expression and/or protein

modification that are likely to be an alternative signaling pathway to activate other macrophages, favoring the immune response^[9].

A direct correlation was observed between the frequency of MN and DNA DI ($P < 0.0001$, $r = 0.9515$). Lymphocyte count was inversely correlated to MN frequency ($P < 0.0001$, $r = -0.6447$) and to DNA DI ($P < 0.0001$, $r = -0.7432$).

The reduction in the DNA DI parameter indicates that CA has a rapid antigenotoxic action against the intense genotoxic damages caused by NMU^[10]; the DNA ID induced by NMU in day 240 reduced to a basal

level in the animals treated with CA only one month after the NMU treatment was discontinued. A reduction in the MN count was also significant in the animals treated with CA. The reduction of these alterations was significantly correlated with an increase in lymphocyte count, which was observed in the CA group. Thus, the reduction of chromosomal and genetic alterations may reflect the presence of a mixed population of lymphocytes. We hypothesize that the CA group may present older lymphocytes which were exposed to MNU and new lymphocytes.

In conclusion, we suggest that CA may have a role as an immune response modulator by inducing the proliferation of leukocyte and erythrocyte progenitors in the bone marrow. We also suggest that CA can protect against NMU-mediated genotoxicity increasing the count of healthy cells in the bloodstream. Further investigations with a large number of animals are necessary to evaluate whether CA could be used in anticancer treatments to restore the hematopoietic system during/after chemotherapy.

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