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The protective effect of *Canova* homeopathic medicine in cyclophosphamide-treated non-human primates

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ABSTRACT

Background: *Canova* activates macrophages and indirectly induces lymphocyte proliferation. Here we evaluated the effects of *Canova* in cyclophosphamide-treated non-human primates.

Methods: Twelve *Cebus apella* were evaluated. Four animals were treated with *Canova* only. Eight animals were treated with two doses of cyclophosphamide (50 mg/kg) and four of these animals received *Canova*. Body weight, biochemistry and hematologic analyses were performed for 40 days. Micronucleus and comet assays were performed for the evaluation of DNA damage.

Results: We observed that cyclophosphamide induced abnormal WBC count in all animals. However, the group treated with cyclophosphamide plus *Canova* presented a higher leukocyte count than that which received only cyclophosphamide. Cyclophosphamide induced micronucleus and DNA damage in all animals. The frequency of these alterations was significantly lower in the *Canova* group than in the group without this medicine.

Conclusions: Our results demonstrated that *Canova* treatment minimizes cyclophosphamide myelotoxicity in *C. apella*.

1. Introduction

Cancer is a leading cause of death worldwide (WHO, 2010). Several protocols using chemotherapic and radiotherapic approaches have been developed for anticancer treatments. However, protecting patients from adverse effects of these intensive therapies has been a goal of clinical oncologists (Bukowski, 1999).

For over 40 years, cyclophosphamide (CP) has been in clinical use for the treatment of malignant and nonmalignant disorders (Hosseinimehr and Karami, 2005). However, CP is an alkylating agent that produces gene mutations, chromosome aberrations, micronuclei (MN) and sister chromatid exchanges (Anderson et al., 1995). CP causes adverse effects especially in the hematopoietic system (primarily represented by leukopenia), and the gastrointestinal tract and also can produce secondary tumors due to its genotoxicity (Schuurman et al., 2005).

New adjuvant therapeutic approaches to known therapies and chemoprotectants, for example glutathione, amifostine and quercetin (Jena et al., 2010), have been developed as methods to protect normal tissues from the toxicity of antineoplastic agents without

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compromising antitumor efficacy and, thus, providing a better quality of life during anticancer treatment (Stolarska et al., 2006).

Canova (CA) is a complex homeopathic medicine indicated for patients whose immune system is depressed. CA treatment seems to enhance an individual's ability to trigger a specific immunologic response against several pathological conditions (Cesar et al., 2008).

It was previously demonstrated that CA treatment induces macrophage activation and indirectly induces lymphocyte proliferation *in vivo* and *in vitro* (Abud et al., 2006; Burbano et al., 2009; Cesar et al., 2008; Da Rocha Piemonte and De Freitas Buchi, 2002; de Oliveira et al., 2006; Lopes et al., 2006; Pereira et al., 2005). Moreover, our research group has previously demonstrated that CA treatment does not induce cytotoxic or genotoxic effects at the chromosomal level *in vitro* (Seligmann et al., 2003).

Due to their phylogenetic proximity to humans, non-human primates offer a useful model for basic research into genetic and immunopathogenesis mechanisms as well as for the development and validation of new therapies for several diseases. Non-human primates are also of particular relevance in evaluating the potential toxicity of drugs and environmental agents. *Cebus apella* is an interesting model for drug studies, since they can be easily housed in Primate Research Centers due to their flexibility, opportunism, adaptability, small size, and with annual reproduction of these animals being possible in primatology centers. Currently, *Cebus* species are frequently used in neuroscience, dentistry, reproduction, and behavioral research (Torres et al., 2010).

Since CA treatment leads to lymphocyte differentiation and proliferation (Cesar et al., 2008), the aim of the present study was to evaluate whether this drug has a protective effect against the toxicity caused by chemotherapy. We analyzed the effects of CA medicine in CP-treated non-human primates, *C. apella*.

2. Materials and methods

2.1. Canova

'Canova do Brazil', a Brazilian company, holds the international patent of this medicine (CANOVA, 2012). Experiments were performed with commercial CA donated by 'Canova do Brazil'.

CA is an aqueous, colorless and odorless solution. The Hahnemannian homeopathic method used to prepare the *Canova* medicine is described in the Brazilian homeopathic pharmacopoeia (CPRFB, 1997). dH units (decimal Hahnemannian) were used. The number before the dH is the number of times the decimal dilution is made; 10 dH would be 1×10^{-10}. 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. In natural medicine, each of these components is used separately in the treatment of different diseases, including facial paralysis, joint pain, gout, uterine and colorectal polyps, and cancer.

2.2. Animals and treatments

Twelve male adult *C. apella* (6–8 years old) were evaluated (2.8–3.4 kg). All animals were born and bred in captivity in Centro Nacional de Primatas (CENP), Pará State, Brazil. The *C. apella* is easily available in CENP.

In this study, the details of animal welfare and steps taken to ameliorate suffering were in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research" (Sir David Weatherall FRS FMedSci's working group, 2006). This study received the approval of the Ethics Committee of Universidade Federal do Pará (PARECER MED002/2007).

Animals were identified with microchips and were individually housed in the CENP. All animals were maintained in aluminum squeeze cages ($80 \times 90 \times 80$ cm) under a natural photoperiod. The animals were fed with fresh fruit, vegetables and commercial food pellets (FOXY Junior Supreme 28% crude protein; PROVIMI, Brazil). Water was given *ad libitum*.

Before the study, animals were confined in individual cages during 40–50 days. Blood, urine and stool tests were performed and the body weight was recorded. The animals received appropriated veterinary care if any abnormality was detected. Animals without standard body weight get specific diet. After 40–50 days, new tests were performed and the animals which presented some health problem were removed from the study (3 of 15 animals). Among the three animals excluded from entry in the study, two animals presented the weight below the average and the other animal presented some hematology parameters suggestive of anemia.

According to a basic veterinary examination, all animals were considered healthy at the time of the first blood sampling. This was confirmed by the animals' behavior as judged by the veterinary check.

The animals were randomly divided into three groups of four *C. apella* each. One group received only CP (CP group), the second group was treated with CA only (CA group) and the third group was treated with both CP and CA (CP + CA group).

The CP and CP + CA groups were treated with 50 mg/kg B.W. CP (Cytoxan®, Bristol–Myers Oncology, USA) dissolved in sterile physiological saline solution on the day of dosing. The CP dose level was determined based on previous studies with nonhuman primates which evaluated different applications of CP (da Costa et al., 2011; Schuurman et al., 2005). All eight animals received CP twice (day 1 and 20). The interval between CP treatments was determined considering the recovery of leukocyte count.

The CA and CP + CA groups received injections of 1.67 μl/g commercial CA (intravenous form) on day 1 and 20. Those in the CP + CA group were administered the CP at the same time. The CA dose level was determined according the study of Moreira et al. (2012) in *C. apella* which evaluated different applications of CA.

The animals were weighed daily and the doses calculated at that time. CP and CA were injected by slow infusion in the right femoral vein of *C. apella* in a single dose (Sharma et al., 1999).

2.3. Animal evaluation

Two days before the infusions (day 1 and 0), blood samples of all animals were collected for the determination of hematologic parameters and for the evaluation of hepatic and renal functions (Fig. 1). These samples were used as base values for these analyses. For genotoxicity analyses, the blood sample of day 0 was used as base values.

During treatment periods, animals were inspected daily and clinical symptoms recorded. For minor manipulation or close observation, physical restraint (squeeze cages) and chemical restraint were used. Daily, the animal were sedated with ketamine hydrochloride (Ketaral, 15 mg/kg), and, then, body weight was determined and about 3 ml of peripheral blood was collected for serum biochemistry and hematologic analyses. The blood samples were obtained from femoral veins and the volume of blood collected was based on each animal's weight in accordance with the "Guide for the care and use of laboratory animals" (National Research Council, 1996).

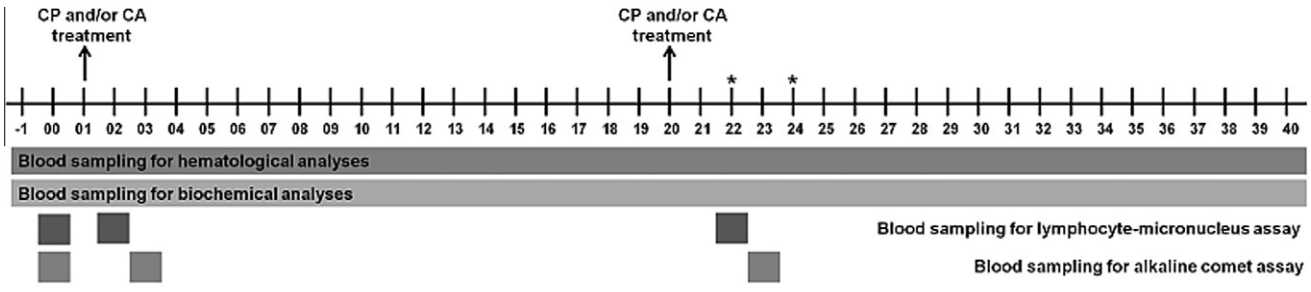

Fig. 1. Time line of drug treatments and blood collections. ∗, Animals from CP-group that were euthanized. CP, cyclophosphamide. CA, *Canova* medicine.

Serum chemistry analysis was performed using Vitros DT60 II Dry Chemistry System (Johnson & Johnson, USA) and included glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, cholesterol, triglyceride, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, lactate dehydrogenase, creatine kinase, amylase, calcium, inorganic phosphorus, sodium, potassium, and chloride. Clinical hematology included red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell (WBC) and differential (segmented neutrophil, lymphocyte, monocyte, eosinophil and basophile) counts. Hematology analyses were performed using an automated blood cell counter (CC-530, Celm, Brazil). Manual WBC differential was obtained by counting 100 leukocytes in Wright-stained blood smear. Reference values for male adult animals were previously described by Riviello and Wirz (2001). Most of the analyses were performed for 40 days (Fig. 1).

Alterations in the hematology parameters, especially WBC and differential, represented major CP adverse effects observed in the laboratory parameters analyzed, as also previously observed (Schuurman et al., 2005). Thus, our results focus mainly on these parameters.

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 CP effects, through micronucleus and comet assays.

2.4. Animal's euthanasia

Animals suffering and with presumed terminal illness due to serious adverse side effects were euthanized. Animals were euthanized by intravenous administration of Ketaral® (Cetamine chloride, 50 mg/kg), Dormonid® (Midazolam, 50 mg/kg) and Methotrimeprazine® (Levomepromazine, 50 mg/kg). After euthanasia, the animals were submitted for necropsy and organs and body fluids were collected for laboratory analysis. Before euthanasia, a blood sample was also collected for comet assay.

2.5. Genotoxicity analyses

To evaluate the *in vivo* genotoxicity of CA medicine, we performed *in vivo* comet and MN assays. These two tests have been successfully combined to determine drug genotoxicity *in vivo* since they presented differences in sensitivity, endpoints measured and the type of data generated (Vasquez, 2010). While DNA damage, measured by the comet assay, cause structural chromosome aberrations following errors of DNA replication, micronucleus arises from acentric fragments, one of the structural aberrations, when the fragment its failed to be included in a daughter nuclei and is left in the cytoplasm (Igarashi et al., 2010). Combining these two assays allow the reduction of animal use in safety testing (Vasquez, 2010).

2.5.1. Lymphocyte-MN assay

We previously observed that the highest CA effect in lymphocyte culture occurs after 72 h. Peripheral blood samples were collected from all *C. apella* 24 h after CP, CA, or CP + CA treatments (day 2 and 21, respectively; Fig. 1). Short-term lymphocyte cultures were initiated according to standard protocol (Preston et al., 1987). Lymphocytes were cultured in HAM-F10 medium (Sigma–Aldrich, USA) supplemented with 19.2% heat-inactivated fetal calf serum, 1% streptomycin, 1% penicillin and 2% (v/v) phytohemagglutinin (PHA, M Form, #10576015, Life Technologies, USA). Lymphocytes were cultured for 72 h (37 °C, humidified atmosphere with 5% CO_2). Forty-four hours after PHA stimulation, 3 μg/ml of cytochalasin B (Sigma–Aldrich, USA) was applied to all cultures. Cytochalasin B is an inhibitor of actin polymerization, which prevents the separation of daughter cells after mitosis and, then, leads to binucleated cells (Carter, 1967). Cultures were harvested 28 h after cytochalasin B addition.

Cellular suspension was centrifuged at 1000 rpm for 5 min. Then, cells were resuspended in a mild hypotonic treatment (KCl 0.075 M) maintained at 4 °C for 3 min. Subsequently, the cells were centrifuged and a methanol/acetic acid (5:1) solution was gently added. The fixation step was repeated twice in methanol/acetic acid (3:1) and finally, cells were deposited onto clean slides. The slides were stained with 10% Giemsa (pH 6.8) for 3–4 min. Slides were mounted and coded prior to the microscopic analysis.

The frequency and the distribution per cell of MN were determined by analyzing 2000 binucleated cells with well-preserved cytoplasm obtained from each animal. The identification of MN was carried out as previously described (Fenech, 1993). MN were identified as structures that appeared separated from the nucleus, were round or oval, showed staining characteristics similar to those of the nuclei, and were smaller in size than one-third of the area of the original nucleus. All analyses were performed in a blind test.

2.5.2. Alkaline comet assay

Considering our previous observation that the highest CA effect in lymphocyte culture occurs after 3 days, peripheral blood samples were collected from *C. apella* for alkaline comet assay (single-cell gel electrophoresis) analysis 72 h after CP, CA or CP + CA treatments (Fig. 1). A blood sample was collected 48 h after CP treatment from one animal of the CP group, due to the process of euthanasia (day 22).

Peripheral blood lymphocytes were isolated by Ficoll density gradient (Hystopaque 1077, Sigma–Aldrich, USA). Before the comet assay, the cell viability was analyzed by trypan blue exclusion. Lymphocytes were stained with 0.25% (w/v) trypan blue (T8154, Sigma–Aldrich, USA). The unstained (viable) cells were counted under light microscopy. One hundred 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 the comet assay.

Alkaline comet assay was performed as described by Singh et al. (1988), with minor modifications. Lymphocytes were mixed with low-melting-point agarose. Slides were prepared in duplicate and 100 nucleoids were screened per sample (50 cells from each duplicate slide) with a fluorescence microscope (Olympus, USA). Undamaged cells appeared as intact nuclei without tails, whereas damaged cells had the appearance of a comet. Comets were classified visually as belonging to one of five classes according to tail size into five classes: 0, undamaged; 1, low damage; 2, medium damage; 3, high damage; and 4, almost all DNA in the tail (maximally damaged). The total number of comets for each class was multiplied by the value of its class, thus creating an arbitrary unit (score). Therefore, the total score for 100 comets can range from 0 (all undamaged) to 400 (all maximally damaged) (Speit and Hartmann, 1999). The DNA damage index (DI) is based on the length of migration and on the amount of DNA in the tail and is considered a sensitive measure of DNA damage.

2.6. Statistical analysis

In the present study, only non-parametric tests were applied. Kruskal–Wallis followed by the Games-Howell post hoc test were performed to compare the body weight, serum biochemistry values, hematological parameters, MN frequencies and DNA DI among CP, CA and CP + CA groups. Friedman was performed to compare MN frequency, number of comets and DNA DI among treatments. The Wilcoxon's test with Bonferroni correction were applied as a post hoc analysis for the Friedman's test. The correlations between WBC or lymphocyte count (using as base value only the analyses of day 0), MN frequency and DNA DI were analyzed by the Spearman's test, in which a Rho (ρ) value below 0.30 was determined as a weak correlation; 0.30–0.70 as a medium correlation; and above 0.70 as a strong correlation. In all analyses, p values less than 0.05 were considered significant.

3. Results

3.1. CP serious adverse side effects in C. apella

In baseline (days 1 and 0), none of hematological and biochemistry parameters, as well as the body weight, differed among groups. During the study, two animals from the CP group were sacrificed on days 22 and 24 due to the presence of serious adverse side effect (Fig. 1).

The two euthanized animals presented reduced WBC count of 2.64 and 1.75 $\times$ 10^3/μL, sleepiness, giddiness, loss of balance, low food consumption, nonspecific gastrointestinal symptoms (diarrhea and vomiting), cutaneous eruptions and caustic and ulcerative oral lesions. Before the sacrifice of these animals, they also presented renal and respiratory failure, hypokalemia, and elevation of alanine aminotransferase, bilirubin, creatinine, and phosphorus levels.

The necropsy showed renal damage with degeneration of adipose tissue, hepatic venoocclusive disease, inflammatory lesions of the gastrointestinal system, atrophy of villi and sterile hemorrhagic cystitis (noninfectious). No sign of pneumonia was observed in the two euthanized animals. These findings suggested that these two animals presented CP intoxication.

In addition, the two euthanized animals presented small herpesvirus lesions on the tongue, lips and/or skin. These two animals presented more lesions than the other two CP-treated animals. In a small number, the herpesvirus lesions were also detected in the animals of CP + CA group. However, after the end of the present study, no animal presented herpesvirus lesions.

Moreover, the surviving animals did not present serious adverse side effects. They were clinically monitored for one year after the end of the experiment and they did not show complications resulting from the CP or CA treatments.

3.2. CA effects in CP-treated C. apella

During the study, the body weight differed among groups on days 7–29 and 32–36 ($p < 0.05$ for all comparisons, Kruskal–Wallis test). The Games-Howell post hoc analyses showed that the body weight was significantly lower in the CP group than in the CA group on all these days ($p < 0.05$). The CP group also presented lower weight than CP + CA group on days 18 and 20 ($p < 0.05$). Moreover, the CP + CA group presented lower weight than the CA group on days 23–29 and 35 ($p < 0.05$) (Fig. 2, Supplementary Table 1).

The CA induced a slight increase of WBC count in the CA group. The main effect of CA on WBC count of *C. apella* was on the 5th day (Fig. 3A, Supplementary Table 2). The WBC count differed among groups on days 4–9, 22–30, and 40 ($p < 0.05$ for all comparisons, Kruskal–Wallis test). The CP group presented a lower WBC count than the CA group on days 4–9, 22–24, 26, 28–30 and 40, and lower than the CP + CA group on days 4, 23–24, 26, 28–29 ($p < 0.05$ for all comparisons, Games-Howell post hoc analysis). Moreover, the CP + CA group presented a lower WBC count than CA group on days 4–7 and 22–30 ($p < 0.05$ for all comparisons). Considering the normal limit of WBC count of 4.3–12.15 $\times$ 10^3/μL (Riviello and Wirz, 2001), no animals from the CA and CP + CA groups presented abnormal WBC count. In the CP group, two animals presented a WBC count below the normal limit on days 4 and 5. The WBC count of these two animals returned to the normal limit before the second CP dose. However, animals from the CP group returned to present abnormal WBC count after the second treatment and two animals were sacrificed due to serious adverse side effects (days 22 and 24; Fig. 3A, Supplementary Table 2).

In the CP and CP + CA groups, the WBC count reduction was due to a significant reduction in the number of lymphocytes and of neutrophils (Fig. 3B and C, Supplementary Table 2). CA was able to induce an increase of lymphocyte count in CP-treated animals since it was possible to observe that lymphocytes were significantly lower in the CP than in the CP + CA group on days 3–22, 24–29 and 32 ($p < 0.05$ for all comparison, Games-Howell post hoc analysis for Kruskal–Wallis test). Moreover, all animals in the CP group and no animals from the CA and CP + CA groups presented abnormal count of lymphocytes.

Neutrophils were also significantly lower in the CP than the CP + CA group on days 9–15, 18–20, 25, 27–30, 33, 35–37, and 39 ($p < 0.05$ for all comparisons, Games-Howell post hoc analysis for Kruskal–Wallis test). The eosinophil counts did not differ between groups. No group presented monocytes and basophils in blood samples.

The erythrocyte count differed among groups on days 2–14, 20–21, 23–39 ($p < 0.05$ for all comparisons, Kruskal–Wallis test). The erythrocyte count was also reduced to abnormal values with CP treatment in CP and CP + CA groups. However, the number of erythrocytes was significantly lower in the CP than the CP + CA group on days 2, 4–9, 34, and 38–39 ($p < 0.05$ for all comparisons, Games-Howell post hoc analysis for Kruskal–Wallis test; Fig. 3D, Supplementary Table 2).

The urea nitrogen level was higher in the CP and CP + CA groups than in the CA group during most of the studied days (Fig. 4A, Supplementary Table 3). The Games-Howell post hoc analysis for Kruskal–Wallis test showed that urea nitrogen level was significantly higher in the CP group than CP + CA group on days 3–6, 8–9, 25–26 ($p < 0.05$ for all comparisons), demonstrating a CA effect on CP-treated animals. Moreover, all animals of the CP group and no animal from the CA presented increased values of urea nitrogen.

The creatinine level differed among groups on several days (Fig. 4B). Creatinine level was significantly higher in the CP group than the CP + CA group on days 25–26 and 33–34 ($p < 0.05$ for all comparisons, Games-Howell post hoc analysis for Kruskal–Wallis test). According to the normal levels of *C. apella* serum chemistry values, only two animals from the CP group presented abnormal values of creatinine. The animals showing elevated values were those that were sacrificed after the second CP treatment.

The phosphorus (Fig. 4C), alanine aminotransferase (Fig. 4D), and total bilirubin (Fig. 4E) levels were significantly higher in the CP and CP + CA groups than in the CA group during several days (Supplementary Table 3). However, no significant effect of CA was observed in CP-treated animals since any difference between CP and CP + CA groups was detected. According to the normal levels of total bilirubin in *C. apella*, only two animals from the CP group presented abnormal values of total bilirubin, as well as creatinine, and were those that were sacrificed after the second CP treatment.

The albumin level was reduced due to CP treatment in CP and CP + CA group compared to CA group on days 30–40 ($p < 0.05$ for

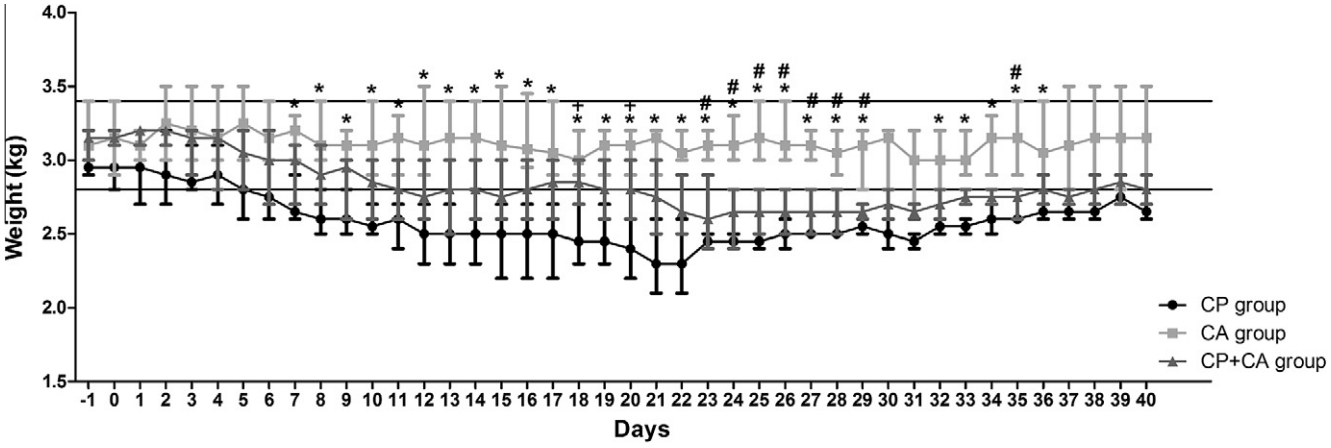

Fig. 2. Median and range of body weight of *Cebus apella* by day. CP group: animals that received 50 mg/kg cyclophosphamide on days 1 and 20. CA group: animals that received only *Canova* medicine. CP + CA group: animals that received *Canova* medicine with 50 mg/kg cyclophosphamide on days 1 and 20. *, Significant difference between CP and CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; +, significant difference between CP and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; #, significant difference between CA and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test. Black line: highest and lowest value observed in the baseline.

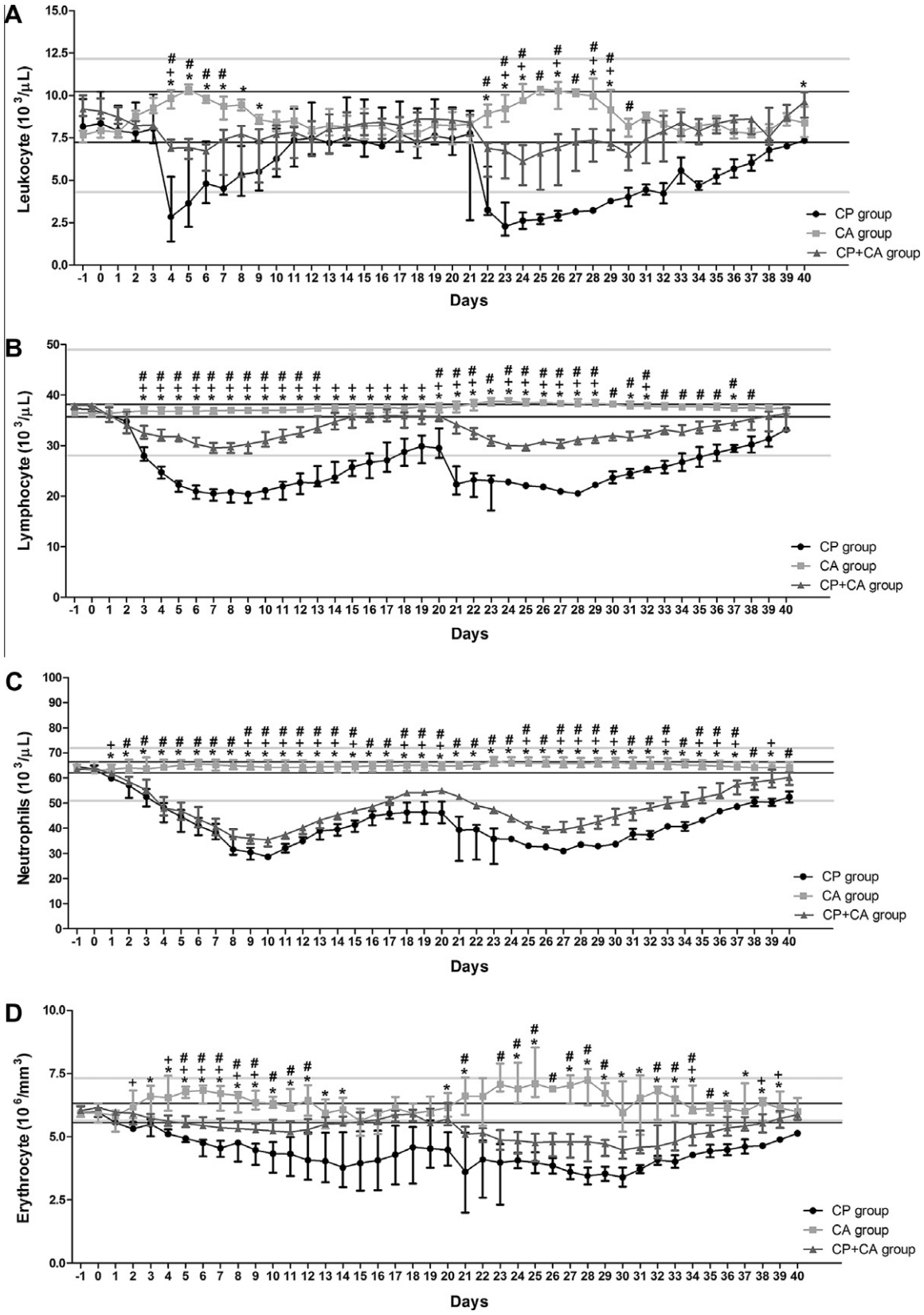

Fig. 3. Median and range of hematological parameters of *Cebus apella* by day. (a) WBC count; (b) lymphocytes; (c) neutrophils; (d) erythrocytes. CP group: animals that received 50 mg/kg cyclophosphamide on days 1 and 20. CA group: animals that received only *Canova* medicine. CP + CA group: animals that received *Canova* medicine with 50 mg/kg cyclophosphamide on days 1 and 20. ∗, Significant difference between CP and CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; +, significant difference between CP and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; #, significant difference between CA and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test. Black line: highest and lowest value observed in the baseline. Gray line: highest and lowest value reported in normal adult *C. apella* by Riviello and Wirz (2001).

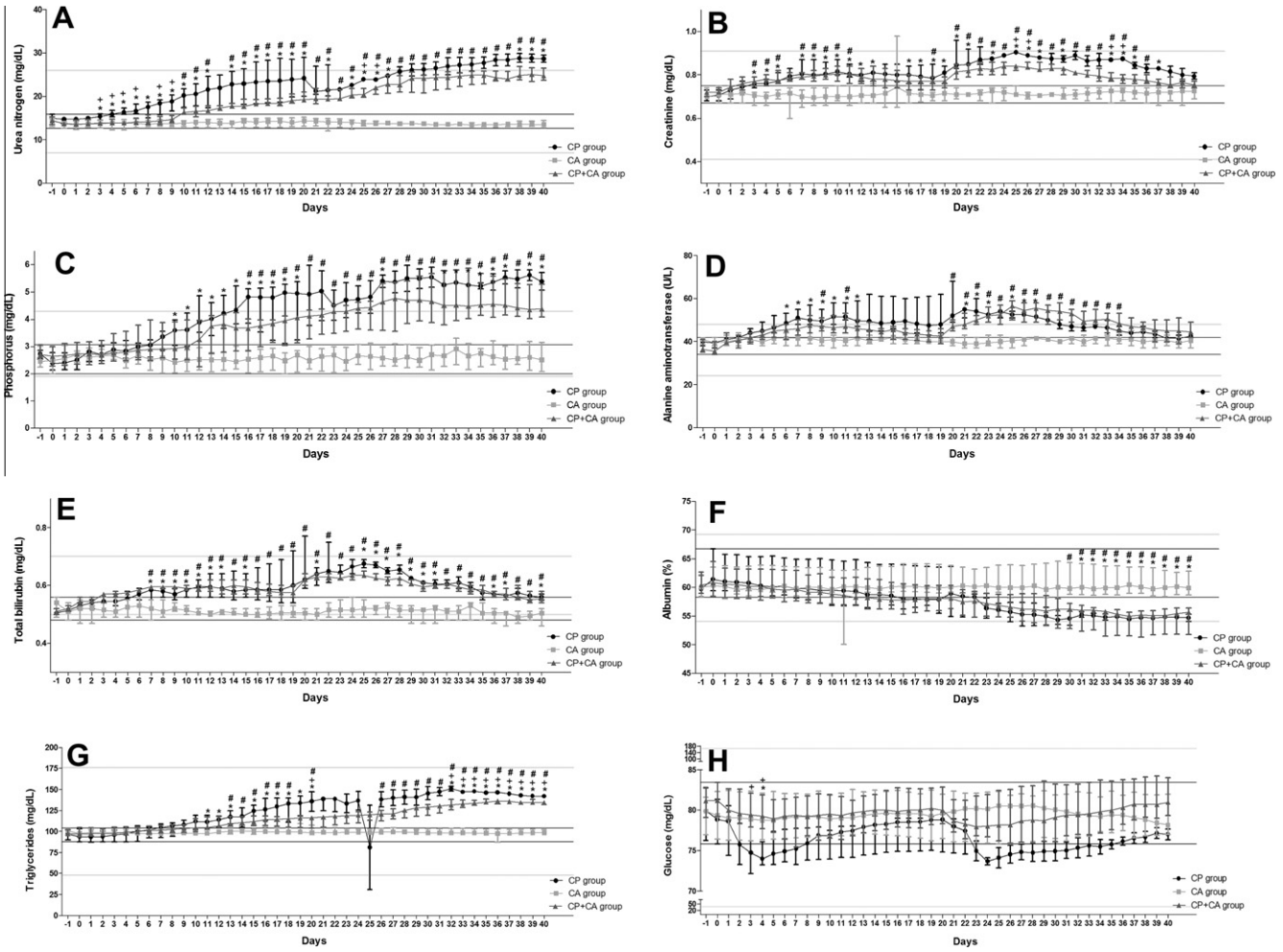

Fig. 4. Median and range of biochemical parameters of *Cebus apella* by day. (a) Urea nitrogen; (b) creatinine; (c) phosphorus; (d) alanine aminotransferase; (e) total bilirubin; (f) albumin; (g) triglycerides; (h) glucose levels. CP group: animals that received 50 mg/kg cyclophosphamide on days 1 and 20. CA group: animals that received only *Canova* medicine. CP + CA group: animals that received *Canova* medicine with 50 mg/kg cyclophosphamide on days 1 and 20. ∗, Significant difference between CP and CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; +, significant difference between CP and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test; #, significant difference between CA and CP + CA groups ($p < 0.05$) by Games-Howell post hoc analysis for Kruskal–Wallis test. Black line: highest and lowest value observed in the baseline. Gray line: highest and lowest value reported in normal adult *C. apella* by Riviello and Wirz (2001).

all comparisons, Games-Howell post hoc analysis for Kruskal–Wallis test; Fig. 4F, Supplementary Table 3). Furthermore, only one animal from the CP group presented abnormal triglyceride level (Fig. 4G) and no animal show abnormal glucose level (Fig. 4H, Supplementary Table 3). The CP group exhibited a significantly higher triglyceride level than the CP + CA group on day 20, and between days 32 and 40 ($p < 0.05$ for all comparisons, Games-Howell post hoc analysis for Kruskal–Wallis test). Moreover, the CP group presented lower glucose than the CP + CA group on days 3 and 4 ($p = <0.05$).

3.3. CA effects on MN frequency and lymphocyte DNA damage in CP-treated C. apella

Table 1 shows the MN distribution in binucleated cells from *C. apella*, before and after the first or the second round of treatments in the CP, the CA and the CP + CA groups. We observed that the distribution of the MN number, the mean MN per 1000 binucleated cells, and the total number of binucleated cells presenting MN, differed among the three days of analyses only in the CP and the CA + CP groups ($p < 0.05$ for all comparisons, Friedman test). However, we were not able to demonstrate any significant difference among treatments after post hoc analyses.

Before treatments, the MN frequency differed among the three groups ($p < 0.05$, Kruskal–Wallis test). The Games-Howell post hoc analyses demonstrated a higher MN frequency in the CP + CA group compared to the CA group ($p < 0.05$) in the baseline. However, lymphocytes presented more frequently one MN by binucleated cell and the number of MN by animal was very small (2–6 MN/1000 binucleated cells) in all groups.

The MN frequency differed among groups after the first ($p < 0.01$, Kruskal–Wallis test) and the second ($p < 0.01$) round of treatments. After the first CP and/or CA treatment, the post hoc analyses showed that the MN frequency was higher in the CP group compared to the CA ($p < 0.001$) and to the CP + CA ($p < 0.001$) groups, and that the CP + CA group presented a higher frequency of MN compared to the CA group ($p = 0.001$). After the second dose of treatment, the CP group also presented a higher MN frequency than the CA ($p < 0.001$) and the CP + CA ($p < 0.01$) groups, and the CP + CA group presented a higher frequency of MN compared to the CA group ($p < 0.01$).

The number of cells presenting 1, 2, or 3 MN and the total number of binucleated cells presenting MN differed among groups after the first and second round of treatments ($p < 0.05$ for all comparisons, Kruskal–Wallis test). The Games-Howell analyses showed that the CP group presented more MN than the CP + CA group,

Table 1
Micronuclei distribution in binucleated cells from *Cebus apella* treated with cyclophosphamide and/or *Canova*.

Treatment	Distribution of MN in BN [median (IR)]			Total of MN	MN/1000 BN [median (IR)]
	1 MN	2 MN	3 MN		
CP group					
Before treatment[a]	2.00 (0.25)	0.50 (0.75)	0.00 (0.00)	3.50 (3.75)	1.75 (0.88)
First treatment	172.50 (9.75)	60.00 (6.00)	25.00 (6.75)	367.00 (13.50)	183.75 (6.75)
Second treatment	181.50 (15.00)[b]	67.50 (6.00)[b]	30.50 (7.00)[b]	408.00 (7.50)[b]	204.00 (3.75)[b]
CA group					
Before treatment[a]	2.00 (0.50)	0.50 (1.00)	0.00 (0.00)	2.00 (0.50)	1.00 (0.25)
First treatment	3.00 (0.5)[d]	0.50 (1.0)[d]	0.00 (0.00)[d]	4.00 (2.50)[d]	2.00 (1.25)[d]
Second treatment	2.50 (1.5)[f]	0.00 (0.5)[f]	0.00 (0.25)[f]	3.50 (2.25)[f]	1.75 (1.13)[f]
CP + CA group					
Before treatment[a]	3.00 (0.25)	1.00 (0.24)	0.00 (0.00)	5.00 (0.25)	2.50 (0.13)[c]
First treatment	67.00 (5.75)[d,e]	42.50 (5.00)[d,e]	9.50 (4.75)[d]	179.00 (29.50)[d,e]	89.75 (14.75)[d,e]
Second treatment	77.50 (11.75)[b,f,g]	47.50 (7.50)[b,f,g]	11.00 (7.50)[b,f]	204.50 (50.25)[b,f,g]	102.25 (25.13)[b,f,g]

MN, micronuclei according Fenech (1993); BN, binucleated cells. IR: interquartile range. 8000 cells were analyzed per treatment for MN score, in which 1 MN represents the binucleated cells presenting one MN, and so on. CP group: animals that received 50 mg/kg cyclophosphamide on days 1 and 20. CA group: animals that received only *Canova* medicine. CP + CA group: animals that received *Canova* medicine with 50 mg/kg cyclophosphamide on days 1 and 20. Peripheral blood samples for lymphocyte-MN assay were collected 24 h after CP, CA, or CP + CA treatments.

[a] Day 0
[b] Significant difference among treatments ($p < 0.05$), intragroup analysis by Friedman test.
[c] Significant difference compared to the CA group in the baseline ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[d] Significant difference compared to the CP group after the first treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[e] Significant difference compared to the CA group after the first treatment ($p < 0.05$), by Games-Howell post hoc analysis. for Kruskal–Wallis test
[f] Significant difference compared to the CP group after the second treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[g] Significant difference compared to the CA group after the second treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.

which presented more MN than the CA group after the first and second treatments ($p < 0.05$ for all comparisons). For the comparison of the number of cells presenting 3 MN between the CA and CP + CA group after the second round of treatment, we did not observe a significant difference.

In addition, the frequency of MN was inversely correlated to WBC and to lymphocyte count in the CP ($p < 0.01$, $\rho = -0.746$; $p < 0.01$, $\rho = -0.818$, respectively; Spearman correlation) and CP + CA ($p < 0.05$, $\rho = -0.669$; $p < 0.001$, $\rho = -0.944$, respectively) groups.

Table 2 shows the DNA damage scores and index by the comet assay in *C. apella* from the CP, CA, and CP + CA groups. We also observed that comet classes 1, 3 and 4 and DNA DI differed among baseline, as well as the first and the second doses of CP in CP and CA + CP groups ($p < 0.05$ for all comparisons, Friedman test). In addition, comet class 2 also differed among the analyzed days in the CP group ($p < 0.05$). However, we were not able to demonstrate any significant difference among treatments after post hoc analyses.

The DNA DI did not differ significantly among groups in the baseline and differed significantly among groups after the first ($p < 0.01$, Kruskal–Wallis test) and second treatments ($p < 0.01$). The Games-Howell post hoc analyses showed that DNA DI was significantly higher in the CP group than the CA group after the first ($p = 0.001$) and second ($p = 0.001$) treatments. Moreover, the CP group presented higher DNA DI than the CP + CA group after the second round of treatment, but this was not statistically significant. Only a tendency to present a higher DNA DI was observed

Table 2
DNA damage scores and index by comet assay in *Cebus apella* treated with cyclophosphamide and/or *Canova*.

Treatment	DNA damage score [median (IR)]					DNA DI [Median (IR)]
	0	1	2	3	4	
CP group						
Before treatment[a]	78.25 (6.25)	12.50 (1.63)	9.00 (3.00)	0.50 (0.38)	0.00 (0.00)	33.50 (8.75)
First treatment	67.00 (2.75)	15.75 (1.13)	14.50 (1.38)	1.75 (0.88)	0.50 (1.13)	53.00 (6.00)
Second treatment	56.50 (3.00)[b]	21.00 (2.38)[b]	14.75 (0.75)[b]	4.00 (1.13)[b]	2.50 (1.13)[b]	74.25 (5.75)[b]
CA group						
Before treatment[a]	79.25 (1.00)	12.25 (2.88)	8.25 (2.88)	0.5 (0.5)	0.00 (0.00)	29.50 (4.63)
First treatment	81.25 (3.88)	11.50 (2.50)	5.75 (2.38)[c]	0.75 (0.63)	0.00 (0.13)	26.25 (5.63)[c]
Second treatment	80.75 (2.00)	12.00 (6.00)	7.75 (2.13)[e]	0.50 (0.38)[e]	0.00 (0.00)[e]	28.25 (4.63)[e]
CP + CA group						
Before treatment[a]	78.00 (1.00)	11.50 (0.38)	9.25 (1.00)	0.75 (0.63)	0.00 (0.00)	33.75 (2.00)
First treatment	71.50 (0.63)	17.00 (1.50)	11.00 (0.63)[d]	1.00 (0.25)	0.00 (0.00)	41.00 (1.50)[d]
Second treatment	65.75 (0.88)[b]	18.75 (1.00)[b]	12.50 (1.90)	1.75 (0.63)[b,e]	0.75 (0.63)[b,e,f]	52.75 (2.13)[b,e,f]

IR, interquartile range; DI, damage index. 400 cells were analyzed per treatment for DNA damage index analysis. CP group: animals that received 50 mg/kg cyclophosphamide on days 1 and 20. CA group: animals that received only *Canova* medicine. CP + CA group: animals that received *Canova* medicine with 50 mg/kg cyclophosphamide on days 1 and 20. Peripheral blood samples for comet assay were collected 72 h after CP, CA, or CP + CA treatments.

[a] Day 0.
[b] Significant difference among treatments ($p < 0.05$), intragroup analysis by Friedman test.
[c] Significant difference compared to the CP group after the first treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[d] Significant difference compared to the CA group after the first treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[e] Significant difference compared to the CP group after the second treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.
[f] Significant difference compared to the CA group after the second treatment ($p < 0.05$), by Games-Howell post hoc analysis for Kruskal–Wallis test.

in the CP group compared to the CP + CA group after the first round of treatment (not statistically significant). In addition, the CP + CA group presented a significantly higher DNA DI than the CA group after the first ($p < 0.01$) and the second ($p < 0.001$) CP treatment.

A higher frequency of comet class 2 was also observed in the CP ($p < 0.05$, Games-Howell post hoc analysis) and CP + CA ($p < 0.05$) groups compared to the CA group after the first treatment. After the second CP treatment, the distribution of comets classes 2, 3 and 4 also increased in the CP group compared to CA group ($p < 0.05$, p = 0.001, $p < 0.05$, respectively). The CP group also presented a higher frequency of comets class 3 and 4 compared to the CP + CA group after the second treatment ($p = 0.009$, $p < 0.05$, respectively). After the second CP treatment, the CP + CA group also presented a higher frequency of comet class 4 compared to the CA group ($p < 0.05$).

In addition, a direct correlation was observed between the frequency of MN and DNA DI in the CP ($p < 0.001$, $\rho = 0.951$; Spearman correlation) and CP + CA ($p < 0.01$, $\rho = 0.761$) groups. Furthermore, the DNA DI was inversely correlated to WBC and lymphocyte count in the CP ($p < 0.01$, $\rho = -0.8$; $p = 0.001$, $\rho = -0.864$, respectively) and CP + CA ($p < 0.05$, $\rho = -0.783$; $p < 0.01$, $\rho = -0.797$, respectively) groups.

4. Discussion

In the present study, the CP + CA group presented body weight and hematology data between CP and CA groups. On the other hand, the serum chemistry values observed in the CP + CA group showed an outcome more close to the CP group and different from the CA group. Thus, at the dose levels given, CA seems to present a reduced effect regarding liver and kidney toxicity when compared to myelotocicity and body weight effect. Since the CP + CA group received CA and CP drugs at the same time and via the same route, we cannot exclude that CA components may interact with CP before the CP exerts its toxic action, reducing the exposure of the animal to CP.

In this study, animals of CP + CA group presented higher number of WBC, lymphocytes, erythrocytes and neutrophils compared to animals treated only with CP. This finding allows some hypothesis. Firstly, CA may up-regulate these cells in non-human primates treated with CP. It was previously reported that CA can increase directly or indirectly the number of lymphocytes (Coelho Moreira et al., 2012; Sato et al., 2005). In addition, Abud et al. (2006) suggested that CA medicine also facilitates the production of erythroid differentiation regulator, which would justify the increase of erythrocytes in the peripheral blood of *C. apella* treated with CP + CA compared to the CP group in some periods of this study. However, further analyses are necessary to understand how CA may act in neutrophils count.

Secondly, CA may have a cytoprotective effect on cells vulnerable to CP effects. This cyprotective effect might also lead to the reduction of DNA damage in the CP + CA group compared to the CP group. CP induces toxicity through its enzymatic conversion to active alkylating intermediates by cytochrome P-450 (CYP) monooxygenases. The CYP2B and 2C subfamily enzymes are mainly responsible for the convertion of CP into its active metabolites acrolein and phosphoramide mustard (Yu et al., 1999), while CYP3A enzymes metabolize CP to dechloroethylcyclophosphamide and chloroacetaldehyde and seem to play a key role in the detoxification of this akylating agent (Yu et al., 1999).

CA is a complex homeopathic medicine and the effect of its compound is not completely understood. It was previously reported that *Asa foetida,* a CA compound, is able to reduce the levels of cytochrome P450 and b5 and restore the antioxidant system in MNU-induced mammary carcinogenesis in rats (Mallikarjuna et al., 2003). On the other hand, *Lycopodium clavatum*, another CA compound, may have potential to inactivate CYP3A4 (Tam et al., 2011). Since some plant extracts are able to modify the expression and activity of CYP enzymes and, thus, may alter the activation or detoxification of CP (Bagchi et al., 2002; Kumarappan et al., 2011; Ray et al., 2001), further studies are necessary to evaluate whether CA has a cytoprotector effect *in vivo* and in its target cells and, then, determine if CA may be used as a chemopreventive agent in the treatment of solid tumors.

Thirdly, CA may inhibit CP-mediated leukocytopenia and lymphocytopenia. Thus, our findings suggest that CA treatment minimizes CP myelotoxicity in *C. apella.* The CA effects on WBC count, especially lymphocytes, may have human therapeutic applications such as restoring the hematopoietic system after chemotherapy for solid tumors.

Concerning serum chemistry values, the highest difference between groups was observed in urea nitrogen and creatinine levels, indicators of glomerular function of the kidney. CP is nephrotoxic besides being urotoxic thereby limiting its clinical utility (Hamsa and Kuttan, 2011). Here, we observed that CP induced an increase in urea nitrogen and creatinine level, as well as other parameters related to renal damage, compared to the pretreatment serum level of *C. apella*. However, the CP group presented a significant increase of this parameter compared to the CP + CA group. Thus, this study shows that CA treatment may have a role in the reduction of CP-induced renal damage. However, additional investigations are necessary to evaluate if CA may act in renal cells.

5. Conclusions

CP induced abnormal WBC count, MN and DNA damage in all animals. However, CA treated animals presented a higher leukocyte count than the animals without this treatment. Moreover, CA-treated animals presented lower frequency of MN and DNA damage than animals without this medicine. Thus, CA treatment minimizes CP myelotoxicity in *C. apella.*

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2012.09.002.

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