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Research Article

Analysis by the Comet Assay with Rattusnovergicus Wistarline Immuno Suppressed with Cyclophosphamide

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Abstract

Cyclophosphamide commonly used in the chemotherapy of cancer patients, is also used in the treatment of some autoimmune diseases. In this work, we established and characterized a model of immune suppression with the cyclophosphamide-alkylating agent in Rattusnorvegicus of the Wistar line and analyzed the immunological responses and the genotoxic action after administration of the alkylating agent. We used 248 rats of the Rattusnorvegicus species of the Wistar lineage, males, with 90 to 120 days of age. The cumulative genotoxic effect of cyclophosphamide is evident in the application of the third dose of immunosuppressant, which coincides with the death of rodents. For the purposes of immune suppression of Rattusnorvegicus from the Wistar line, from the third inoculation of the drug, concentrations below 50 mg/kg are advisable to avoid massive rodent death.

Keywords: Immunotoxicology; Cyclophosphamide; Model of immunosuppression; Immunological tests; Genotoxic effects

Abbreviations

CP: Cyclophosphamide; DNA: Deoxyribonucleic acid; FDA: Food and Drug Administration; RNA: Ribonucleic acid

Introduction

Immuno toxicologyis defined as being the science that studies the deleterious action of xenobiotics on the immune system. Being one of the most recent branches of toxicology, its beginning is related to the clinical scenario of the 1960s. In this period, we observed the introduction and extensive use of potent immunosuppressive drugs, resulting in the first descriptions of adverse effects caused by these new treatments and, consequently, stimulating interest in the area of immune suppression induced by xenobiotics [1]. Early and accurate evaluation of immunotoxicity and genotoxicity is crucial. However, there are few in vitro models for immunosuppressive evaluation [2].

Cyclophosphamide (CP) is a cytotoxic drug that can suppress both humoral and cellular immunity. Combining traditional medicinal herbs and chemotherapy drugs are used to improve immunity and quality of life performance status [3]. Many studies have been carried out in this context, as we can see below.

CP is a widely used antineoplastic drug with broad-spectrum anti-canceractivity. Is an alkylating agent, which adds an alkyl group to the guanine base of DNA. CP isamong the most utilized drugs in chemotherapy, with Food and Drug Administration (FDA) approved indications in many different types of cancers including breast cancer, leukemia, Hodgkins and non-Hodgkins lymphoma, multiplemyeloma, neuroblastoma, retinoblastoma, and ovarian cancer. However, CP has severe and life-threateningadverse effects. The major toxic side effect of CP is the acute and transient inhibition of hematopoiesis, primarily caused by damage to rapidly proliferating hematopoietic progenitors and their mature progeny leading to leukopenia [4].

In another study, a comparison was made between the variability and reliability of the spleen, thymus and peripheral and histological lymph nodes in immunotoxicity induced by CP in rats [5].

Studies related to the genotoxicity of CP are also reported in the literature, for example [6]. Comment that CP causes cytotoxicity to normal cells in spite of its effective anticanceralkylating agent.The active metabolites of cyclophosphamide, for example, phosphoramide mustard andacrolein, are responsible for accumulation of reactive oxygenspecies resulting in fragmentation of the DNA strand and anincreasing in mutagenic DNA effects. The activated CP metabolites are responsible for inducing damage to DNA, RNA, proteins, and cytoplasmic membranes.

It was also related this drug also exhibits significant immunosuppressive activity andis used to treat autoimmune diseases as well as for renal and bone marrow transplantations [7].

Due to the need to know more about the immune system, experimental models of immune suppression have been established for many years, serving as an important tool for research related to the development of techniques for measuring the immune response, the evaluation of new substances aimed at combating immune depression, in addition to allowing the experimental studies of organ transplantation and also of pathological conditions potentiated by the suppression of the immune system such as cancer [8-10].

In this work, we established and characterized a model of immune suppression with the CP alkylating agent in Rattusnorvegicus of the Wistar line and analyzed the immunological responses and the genotoxic action after administration of the alkylating agent.

Material and Methods

Animals

We used 248 rats of the Rattusnorvegicus species of the Wistar lineage, males (0,20-0,25 kg), with 90 to 120 days of age from the

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Biological Sciences Institute-Universidade Federal do Pará. The animals were kept under standard laboratory conditions and each species received adequate feeding. This research project was approved by the Research Ethics Committee with Experimental Animals of the Federal University of Pará (CEPAE) (002/2007).

Dosing and application of CP

In this project, CP was used in the form of Cytoxan®, ampoules containing a 1g, (Bristol – Meyers Oncology, Princenton, NJ). For the immunosuppressive tests in Rattusnorvegicus of the Wistar line, 185 animals were used in 4 experimental phaseswhich were given in the first and second phases the dose of 50 mg/kg, and in the third and fourth phase the dose of 25mg/Kg CP [10]. In each phase 1 animal / day of each group was euthanized in order to obtain the relative weight and the cellularity of the organs. CP was suspended in saline and injected intraperitoneally into the lower left quadrant of the abdomen, since there are no vital organs in this area except the small intestine. Animals of the control group received the same volume in saline solution. CP solutions were prepared on the day of inoculation.

Anesthesia and euthanasia of animals

Rattusnorvegicus of the Wistar lineage were anesthetized with Vetanarcol (Ketamine Hydrochloride 50mg/ml - Sigma®) 0,5ml/kg Kenzol (Xylazine Hydrochloride, 20mg/ml - Sigma®). The dosage corresponded to 1.8 ml/kg of Vetanarcol and 0.5 ml/kg of Kenzol. Rattusnorvegicus euthanasia of the Wistar lineage was performed by inhalation of a lethal dose of diethyl ether, strictly following the international principles for biomedical research involving animals [11].

Relative weight of organs and cellularity in wistar rats rattusnorvegicus

In Wistar rats, the relative weight (body weight / body weight in g) of the spleen, kidney, thymus and liver was analyzed with the use of a precision scale. The cellularity was evaluated from the cell suspension prepared in RPMI-1640 culture medium, from the bone marrow (femoral), the spleen and the thymus, and counted using the Neubauer chamber [12].

Evaluation of humoral immunity functions in wistar rats rattusnorvegicus

CP-treated rodents and controls received 0.2 ml of 10% SRBC (sheep erythrocytes -sheep red blood cells) via the intraperitoneal route prior to the evaluation of the following parameters:

Hemoagglutination

In order to collect the blood for analysis, at the time of euthanasia, the animals were anesthetized according to the protocol described above, and xiphopovial laparotomy was performed for posterior puncture of the Cava Abdominal Vein with 30x8 needle, coupled in a 3 mL disposable syringe.Syringes and individual needles were used for each animal. This parameter was conducted according to the protocol [13]. with modifications [8].

The blood was stored in Vacutainer tube, centrifuged at 4000 rpm for 10 minutes and stored in a refrigerator at 2 to 8°C. Serial dilutions of serum samples were performed in 50μl of PBS (phosphate buffer, pH 7.2) in 96 micro-titrations mixed with 50μl of 1% SRBC suspended in PBS. After mixing, the containers were kept at room temperature for 2 hours. The titre value of the antibody was determined in the most diluted serum that had visible hemagglutination.

Assay on plate-forming cells in wistar rats rattusnorvegicus

The Plate-Forming Cell (PFC) assaywas performed using the method [12]. 0.2 ml of 10% SRBC prepared in saline was injected intraperitoneally. The animals were submitted to euthanasia from the first day for 107 days of immunization.After removal of the spleen, a 106 cell/ml suspension was made from that organ in RPMI-1640 medium. To analyze the PFC, SRBC was prepared at a cell density of 5x108 cells/ml in PBS. 1 ml of SRBC and 0.5 ml of the diluted guinea pig complement (1 ml of serum + 1 ml saline) were added to 1 ml of the spleen cell suspension.

Cunningham's chambers were prepared using pieces of glass, the mouths were covered and marked on both sides. The cells were filled with the volume of the mixture, sealed with gelatinous oil and incubated at 37°C for one hour. Plates were counted on optical microscopy as the PFC unit per 106 cells of the spleen. The other organs, thymus and liver, followed the same protocol.

Hemolysis Test

In the analysis of the hemolysis test, the method [14]. was modified [8]. The spleen was removed to give a suspension of 10x106 cells/ml PBS. One milliliter of SRBC (0.2%) and 1 ml guinea pig serum (10%) were mixed with the suspension and incubated at 37°C for 1 hour. After centrifugation at 3,000 rpm for 3 minutes the optical density of the supernatant was measured at 413 nm using a spectrophotometer (Eppendorf Biophotometer). The other organs, thymus and liver, followed the same protocol.

Evaluation of the genotoxic effects of cyclophosphamide

Cometassay: The alkaline comet assay was performed according to the work [15]. with small changes [16]. Blood samples (50 ul) were placed in 5 ml anticoagulant (sodium heparin, 25000 UI, Liquaemin®). Blood cell suspensions (5 ul) were soaked in 95 ml of 0.75% low melting agarose (Gibco BRL) and spread on pre-coated agarose microscope slides. After solidification, the slides were placed in lysis buffer (2.5 mol / L NaCl, 100 mmol / L EDTA, and 10 mmol / L Tris, pH 10.0), added fresh with 1% Triton X-100 (Sigma) and 10% DMSO for 48 h at 4. The slides were subsequently incubated in alkaline buffer (300 mmol/l NaOH and 1 mmol / L EDTA, pH> 13) for 20 min at 4°C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform the DNA electrophoresis. The slides were then neutralized (0.4 mol/L Tris, pH 7.5), stained with silver, and visualized under a microscope. 100 cells were randomly selected and then analyzed (50 cells from each of the two replicate slides) from each animal. The cells were also visually classified according to tail size in five classes, ranging from undamaged (0) to a maximum of damaged (4), resulting in a single point of DNA damage for each animal and, consequently, for each group studied. The Damage Index (ID) range from 0 (completely intact, 100 cells×0) 400 (Maximum damaged, 100×4 cells). The lesion frequency (%) was calculated based on the tail number against cells without tail.

According to [17], the comet test has five categories for classification of results, shown in (Figure 1).

Results and Discusion

Immuno suppression was performed in four stages, where the

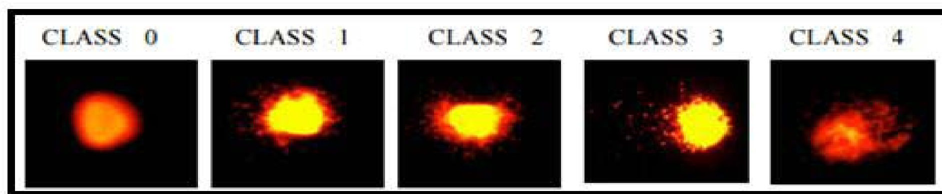

Figure 1: Classes of Comets and their respective scares in the determination of DNA Damage. Classification of DNA damage (genotoxic effect) detected by the Cometa Assay.

Table 1: Effect of treatment with cyclophosphamide on the weight of organs in Rattus norvegicus of the Wistar[a] Lineage.

	Relative organ weight (Mean ± standard deviation) in grams			
Treatment	Spleen	Thymus	Liver	Kidney
Control[b]	0,42±0,41	0,12±0,32	4,2±0,29	0,07±0,37
CP (1 dose 50 mg/kg)	0,19±0,09[c]	0,04±0,17[c]	4,0±0,29	0,06±0,06
CP (2 dose 50 mg/kg)	0,14±0,12[c]	0,03±0,25[c]	3,8±1,54	0,05±0,12
CP (3 dose 25 mg/kg)	0,14±0,23[c]	0,03±0,37[c]	2,1±2,33[c]	0,06±0,09

[a]Values are means and standard deviations of five rats euthanized between the first and fifth day after CP administration.
[b]The values are means and standard deviations of 63 negative control rats.
[c]P <0.05 when compared to the control group.

Table 2: Effect of treatment with cyclophosphamide on the cellularity of the lymphoid organs of Rattus norvegicus of the Wistar[a] Lineage.

	Relative organ weight (Mean standard deviation) in grams		
Treatment	Spleen	Thymus	Liver
Control[b]	305,44±14,57	62,81±9,92	21,33±1,92
CP (1 dose 50 mg/kg)	47,22±9,76[c]	16,44±5,78[c]	13,04±1,67[c]
CP (2 dose 50 mg/kg)	40,41±0,12[c]	12,59±6,65[c]	10,81±1,49[c]
CP (3 dose 25 mg/kg)	39,88±0,23[c]	12,26±5,74[c]	9,15±1,22[c]

[a]Values are means and standard deviations of five rats euthanized between the first and fifth day after CP administration.
[b]The values are means and standard deviations of 63 negative control rats.
[c]P <0.01 when compared to the control group.

animals were divided into two groups, one with CP, treated group (185 rats) and the other without CP, untreated group (63 rats). The first phase lasted for 28 days from day zero, when the first dose of CP (50 mg/kg) was administered. During this period, one animal from each group was euthanized daily in order to obtain the relative weight and cellularity of the organs. On the third and fourth days after inoculation, the immunosuppressive parameters used were the lowest. There was no death after intoxication at this stage. The averages and standard deviations of these parameters are summarized in (Table1,Table 2).

In the second phase CP (50mg/kg) was again administered to the animals at 28 days after the first administration of the drug, since in this time the rats recovered the immune response capacity by the methodology used. One hundred and fifty-seven rats were left from the treated group of the first phase of the experiment. It was observed by the parameters of cellularity and relative body weight that the major immunosuppressive effect of the drug occurred on the second day and was stable until the seventh day.As in the first phase of the experiment, one animal from each group was euthanized daily. At 35 days after administration of the second dose (63 days after administration of the first dose), Wistar rats recovered their immune capacity (Table1,Table 2).

In the third phase of the experiment, the remaining 122 Wistar rats received the third injection of CP, at half the concentration (25mg/ kg) administered at 63 days from day zero. In this phase, on the first day the rats presented the lowest immune depression parameters, this low cellularity remained stable for 12 days, during that period 13 rodents died due to intoxication. Regardless of the sporadic death of rats, every day an animal from the treated group was euthanized, animals from the control group were not used as the values of cellularity and relative body weight were established. After 54 days, the remaining 55 rats from the treated group had immune recovery (117 days after the first administration). The results were summarized in (Table 1, Table 2).

CP was given for the fourth time, 117 days after the start of the experiment, again at half the initial concentration (25 mg/kg) and 17 of the 55 remaining rodents from the third phase of the experiment died between the second and fifth day after inoculation. At this stage of the experiment no animal was euthanized. The relative weight of organs and cellularity was not evaluated in the dead rodents, since the exact moment of death could not be defined. Thus, of the remaining 38 rodents throughout the experiment were monitored for six months and then euthanized.

No significant body weight gain was detected among the animals used in this project. The relative weight of the spleen and thymus

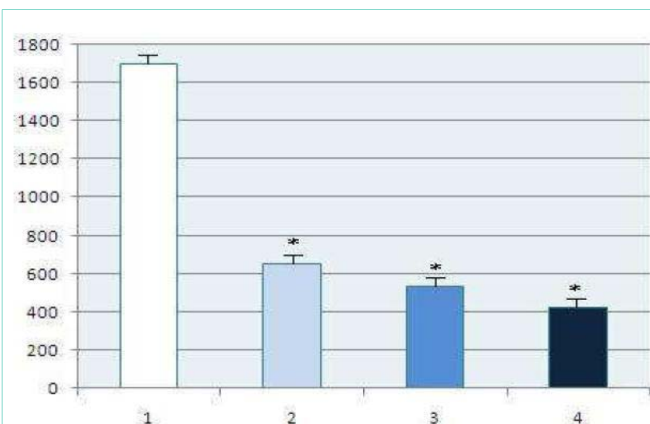

Figure 2: Suppression of humoral immunity induced by cyclophosphamide analyzed by assay on plaque forming cells. Values are means and standard deviations of five rats euthanized between the first and fifth day after cyclophosphamide administration.
* P<0,05 quando comparado com o grupo controle.

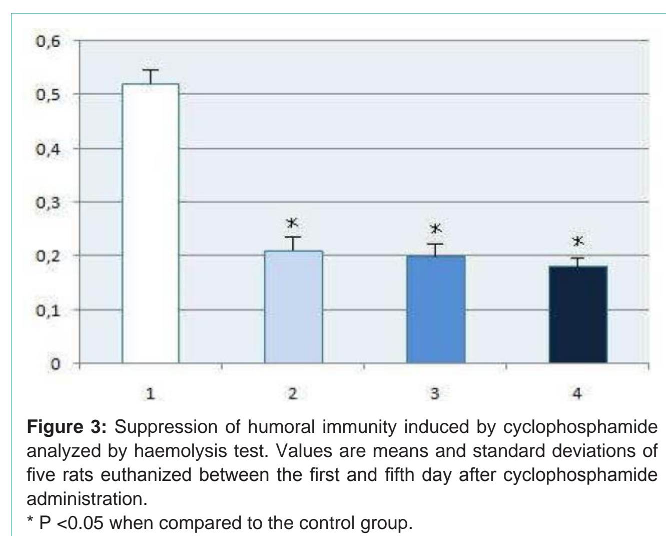

Figure 3: Suppression of humoral immunity induced by cyclophosphamide analyzed by haemolysis test. Values are means and standard deviations of five rats euthanized between the first and fifth day after cyclophosphamide administration.
* P <0.05 when compared to the control group.

of the CP-treated group was significantly lower (P <0.05) when purchased with the control group (treated with standard saline) in the three phases of the experiment (Table 1). For the liver, the relative weight was only significantly lower compared to the control only from the third administration of CP (Table 1). There was no significant reduction of weight in the kidneys of the CP treated animals in relation to the control group (P> 0.05).

Treatment with CP also induced a decrease in cellularity of the bone marrow, spleen and thymus (P <0.001) when compared to the control group of animals (Table 2). Administration of CP induced a significant reduction in all humoral immunity parameters of the treated animals compared to the control group (P <0.05). As previously described, such parameters include: antibody titers (Table 3), PFC formation (Figure 2) and hemolysis test (Figure 3).

The relative weight of the spleen, kidney, thymus and liver and the cellularity of the bone marrow, spleen and thymus were analyzed as parameters for evaluation of immune suppression. Although immunosuppressive experiments in rodents are frequent in the literature, using CP, we did not find an experimental design similar to the one performed in this project [18].

In the present study, the use of the same concentration of CP (a single dose of 50 mg/kg) was used [8,10] and evaluated the same immunosuppressive parameters used in this experiment in Swiss albino mice. Similarly to our results, in the first and second administration of CP in Wistar rats, only the spleen and thymus had a significant decrease in their relative weight.

In the experiments conducted with CP (15 mg/kg) in Wistar rats, evaluated the effect of this drug on the spleen and also found a significant reduction in their relative weight. In the third application of the drug (25 mg/kg), the kidneys and liver also showed a decrease in relative weight, which coincides with the onset of rodent death, probably a systemic intoxication has caused this phenomenon and not the immunosuppressive effect of CP.For this reason, in the fourth CP administration we maintained the same concentration (25 mg/kg), but rodent death increased from 13 to 17 animals, this observation reinforces our hypothesis that there was lethal intoxication of Wistar rats and that the animals did not died from any type of infection. Studies in patients reveal that CP induces cardiovascular toxicity, which can range from arrhythmias to fatal conditions [19].

Regarding organ cellularity, CP treatment also induced a significant decrease in the number of bone marrow, spleen and thymus cells when compared to the control group. Our results are also similar to those found in mice treated with the same concentration of CP as in this experiment [8,20]. An interesting observation is that, independent of Rattusnorvegicus of the Wistar lineage, it is a rodent of greater body weight than the Swiss albino mouse, the cellularity of its organs is not superior.

Administration of CP also induced a significant reduction in all humoral immunity parameters of treated animals relative to the control group. The suppressive effect of CP on antibody titer had values similar to those found in mice [8] and in humans [21].

The PFC assay, which is one of the best tests to predict immune toxicity in mice, showed a significant reduction in humoral immunity in all three CP administrations evaluated in this project. The values found were similar to those described in Swiss albino mice intoxicated with CP [22].

The third trial evaluating the decrease in humoral immunity used in this project was the hemolysis test. There are only two reports in the literature that use this test to evaluate the immunotoxicity of CP [8], and in the same way as the previously discussed assays, the values found in this work with Wistar rats for the hemolysis test are also similar to those in the literature with Swiss mice at the same concentration of CP.

In the immunosuppressive tests in Rattusnorvegicus of the Wistar line we can observe that in the third administered dose of CP (25 mg/kg) the values of all experiments performed are similar, from the point of view of the immune function, than those of the second and the first dose, although the CP concentration administered in each of the first two injections of the drug was double (50 mg/kg).

These results reveal that although there is an increase in cellularity and relative weight of lymphoid organs, which is numerically equivalent to that of control mice, the recovery of the immune system is not complete. In addition, the results lead us to conclude that from the third administration of CP (25 mg/kg), when rats start to suffer lethal toxicity, no higher concentrations of the drug are required to suppress the rodent immune system. A lower concentration such as 25 mg/kg is sufficient to keep Rattusnorvegicus of the Wistar line immune compromised.

Our hypothesis that there is no complete recovery of immune function and that CP induces collateral toxicity can be seen by observing that the recovery periods of the normality, cellularity and relative lymphoid organs in rodents occurred in a time interval from the first dose.In the third dose, although the rats received half the amount of CP, the recovery time was 54 days compared to the 35 days of the second dose and 28 days of the first administration.

The comet assay allowed the detection of genomic damage in the nucleus of blood cells exposed to the different doses of CP, which were 50mg/kg in the first two phases and 25mg/ kg in the last two phases of the experiment. (Figure 4) shows the standard deviation of the

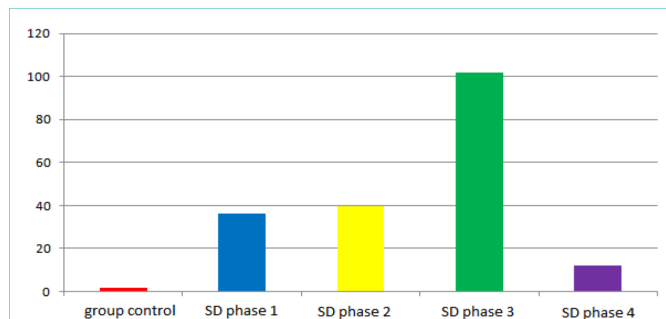

Figure 4: Analysis of the standard deviation of the control group and of each phase of the treatment with cyclophosphamide related to the number of days of *Rattus norvegicus* of the Wistar Line.

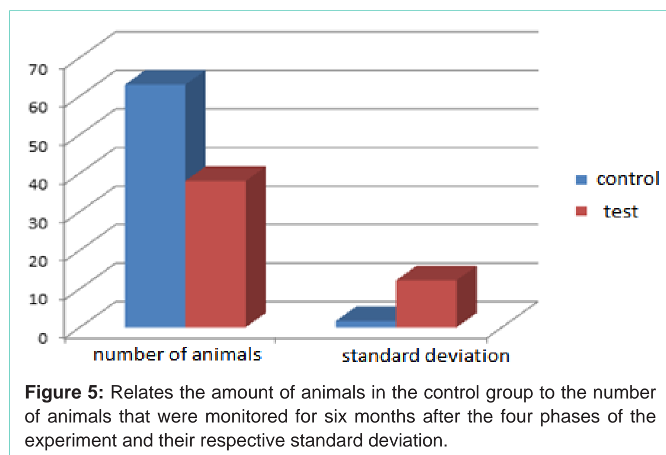

Figure 5: Relates the amount of animals in the control group to the number of animals that were monitored for six months after the four phases of the experiment and their respective standard deviation.

control group and the groups that participated in the CP treatment.

The genome damage index observed in the nucleus of the blood cells constituting the negative control group with 63 animals was 32. The standard deviation of the fourth phase participant group, with 38 animals whose animals were followed for another six months, was 67. Although the number of animals in the control group is greater than those in the fourth phase, we can correlate their respective standard deviations ± 1.68 and ± 12.24 (Figure 5)

Administration of CP significantly increased the DNA damage index and caused a genotoxic effect, which was evident when we analyzed the blood cells through the comet assay (Figure 6)

In Rattusnorvegicus of the Wistarline the three administrations of CP significantly increased the damage index when we evaluated the comet assay in experimental animals, provoking genotoxic effect. This effect was expected, since CP is used as a positive control in several genotoxicity studies described in the literature [23-27].

Although the third administration of CP occurred at half the concentration (25 mg/kg) of the two initial inoculations, the damage index did not correspond to half of the damage index data from the first and second administration. This observation suggests that there is a cumulative genotoxic effect at each dose of CP applied.

Conclusion

The cumulative genotoxic effect of CP is evident in the application of the third dose of immunosuppressant, which coincides with the death of rodents. This effect is potentiated in the fourth inoculation of CP when the number of rodents killed by intoxication increased from 13 to 17 in the third inoculation, although both CP administrations had the same concentration (25 mg/kg). For the purposes of immune suppression of Rattusnorvegicus from the Wistar line, from the third inoculation of the drug, concentrations below 50mg/kg are advisable to avoid massive rodent death.

Table 3: Suppressive effect of cyclophosphamide on the titration of antibodies in Rattus norvegicus of the Wistar Lineage.

Treatment	Title
Control	1.052778
CP (1 dose 50 mg/kg)	01:04
CP (2 dose 50 mg/kg)	01:02
CP (3 dose 25 mg/kg)	01:01

Values referring to the fifth day after inoculation of CP.

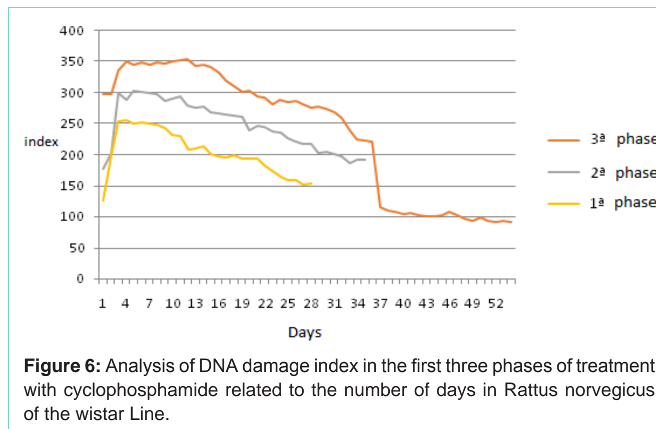

Figure 6: Analysis of DNA damage index in the first three phases of treatment with cyclophosphamide related to the number of days in Rattus norvegicus of the wistar Line.

References

1. Costa RN. Avaliação do efeito imunossupressor mediado pela dexametasona ciclofosfamida e talidomida no ensaio do linfonodo popliteal em ratos. 68f. Monografia Universidade do Rio de Janeiro. 2001; 20: 226-228.

2. Zhao Y, Hao C, Zhai R, Bao L, Wang D, Li Y, et al. Effects of cyclophosphamide on the phenotypes and functions of THP-1 cells. Environ Toxicol Phamacol. 2019; 70: 103201.

3. Shirani K, Hassani FV, Razavi-Azarkhiavi K, Heidari S, Zanjani BR, Karimi G. Phytoterapy of cyclophosphamide-induced immunosuppression. Environ Toxicol Pharmacol. 2015; 39: 1267-1275.

4. Sun-A I, Ki-Hyang K, Hee-Suk K, Ki-Hwa L, Eunju S, Seon-Gil D, et al. Processed Aloe vera gel ameliorates cyclophosphamide-induced immunotoxicity. Int J Mol Sci. 2014; 15: 19342-19354.

5. Lapointe JM, Valdez RA, Ryan AM, Haley PJ. Evaluation on the utility of popliteal lymph node examination in a cyclophosphamide model of immunotocicity in the rat. J Immunotoxicol. 2016; 13: 449-452.

6. Aly FM, Othman A, Haridy MA. Protective effects of fullerene C60 nanoparticles and virgin olive oil against genotoxicity induced by cyclophosphamide in rats. Oxid Med Cell Longev. 2018; 2018: 1261356.

7. Habibi E, Shokrzadeh M, Ahmadi A, Chabra A, Naghshvar F, Keshavarz-Maleki R. Genoptrotective effects of Origanum vulgare ethanolic extract against cyclophosphamide-induced genotoxicity in mouse bone marrow cells. Pharm Biol. 2015; 53: 92-97.

8. Bin-Hafeez B, Ahmad I, Haque R, Raisuddin S. Protective effect of Cassia occidentalis L. On cyclophosphamide-induced suppression of humoral immunity in mice. J Ethnopharmacology. 2001; 75: 13-8.

9. Garcia M, Sertório SP, Alves GJ, Chate SC, Carneiro R, Lallo MA. Uso da

ciclofosfamida em modelos de imunodepressão experimental em ovinos. Pesq Vet Bras. 2004; 24: 115-119.

10. Ferreira SG, Pelliciari-Garcia RA, Takahashi-Hyodo SA, Rodrigues AC, Amaral FG, Berra CM, et al. Effects of melatonin on DNA damage induced by cyclophosphamide in rats. Braz J Med Biol Res. 2013; 46: 278-286.

11. Council for International Organizations of Medical Sciences, 2012. International Guiding Principles for Biomedical Research Involving Animals. 2012.

12. Raisuddin S, Zaidi SI, Singh KP, Ray PK. Effect of subchronic aflatoxin exposure on growth and progression of Ehrlich's ascites tumor in mice. Drug Chem Toxicol. 1991; 14: 185-206.

13. Mungantiwar AA, Nair AM, Shinde UA, Dikshit VJ, Saraf MN, Thakur VS, et al. Studies on the immunomodulatory effects of B. diffusa alkaloid fraction. J Ethnopharmacol. 1999; 65: 125-131.

14. Simpson MA, Gozo JJ. Spectrophotometric determination of lymphocyte mediated sheep red blood cell hemolysis in vitro. J Immunol Methods. 1978; 21: 159-165.

15. Speit G, Hartmann A. The comet assay. A sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol. 2006; 314: 275-286.

16. Picada JN, Flores DG, Zettler CG, Marroni NP, Roesler R, Henriques JA. DNA damage in brain cells of mice treated with an oxidized form of apomorphine. Brain Res Mol Brain Res. 2003; 114: 80-85.

17. Kumaravel TS, Vilhar B, Faux SP, Jha AN. Comet assay measurements a perspective. Cell Biol Toxicol. 2009; 25: 53-64.

18. Dantas JA, Ambiel CR, Cuman KN, Baroni S, Bersani-Amado CA. Valores de referência de alguns parâmetros fisiológicos de ratos do Biotério Central da Universidade Estadual de Maringá, Estado do Paraná. Acta Sci Health Sci. 2006; 28: 165-170.

19. Viale PH, Yamamoto DS. Cardiovascular toxicity associated witch cancer treatment. Clin J Oncol Nurs. 2008; 12: 627-638.

20. Tharakan ST, Kuttan G, Kuttan R, Kesavan M, Austin S, Rajagopalan K. Effect of AC II, an herbal formulation in cyclophosphamide-induced immunosuppression in BALB/c mice- Implication in HIV treatment. Immunol Invest. 2007; 36: 147-157.

21. Vlachoyiannopoulos PG, Toya SP, Katsfis G, Zintzaras E, Tzioufas AG, Moutsopoulos HM. Upregulation of antiphospholipd antibodies following cyclophosphamide therapy in patients with systemic lupus erythematous. J Rheumatol. 2008; 35: 1768- 1775.

22. Ladics GS. Primary immune response to sheep red blood cells (SRBC) as the conventional T-cell dependent antibody response (TDAR) test. J Immunotoxicol. 2007; 4: 149-152.

23. Borroto JI, Creus A, Marcos R. Genotoxic evaluation of the furylethylene derivative 1-(5-bromofur-2-yl)-2-nitroethene in cultured human lymphocytes. Mutat Res. 2002; 519: 179-185.

24. Acevedo HR, Rojas MD, Arceo SD, Soto Hernández M, Martínez Vasquez M, Terrazas T, et al. Effect of 6-nonadecyl salicylic acid and its methyl ester on the induction of micronuclei in polychromatic erythrocytes in mouse peripheral blood. Mutat Res. 2006; 609: 43-46.

25. Selvakumar E, Prahalathan C, Sudharsan PT, Varalakshmi P. Protective effect of lipoic acid on micronuclei induction by cyclophosphamide. Arch Toxicol. 2006; 80: 115-119.

26. Oliveira-Martins CR, Grisolia CK. Determination of micronucleus frequency by acridine orange fluorescent staining in peripheral blood reticulocytes of mice treated topically with different lubricant oils and cyclophosphamide. Genet Mol Res. 2007; 30: 566-574.

27. Vikram A, Tripathi DN, Ramarao P, Jena GB. Evaluation of sptreptozotocin genotoxicity in rats from different ages using the micronucleus assay. Regul Toxicol Pharmacol. 2008; 52: 147-157.