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Efecto con la suplementación de vitamina C sobre la neutropenia inducida por ciclofosfamida en ratones

The effect of vitamin C supplementation on neutropenia induced by cyclophosphamide in mice

ABSTRACT

Neutropenia is one of the adverse effects caused by the administration of chemotherapy drugs. The present study aimed at investigating the effect of vitamin C supplementation in a model of immunosuppression induced by cyclophosphamide in mice. Vitamin C supplementation (50 mg/kg/day) administered intraperitoneally (i.p.) for 7 consecutive days in adult Swiss albino female. Mice were divided into four groups (n= 8/group): 1. Control (only distilled water i.p.), 2. Cyclophosphamide group (cyclophosphamide i.p. 150 and 100 mg/kg on days 1 and 4, respectively and distilled water daily), 3. Vitamin C group (Vitamin C 50 mg/kg i.p. and distilled water daily), and 4. Cyclophosphamide and Vitamin C group (cyclophosphamide i.p. 150 and 100 mg/kg on days 1 and 4, respectively and vitamin C 50 mg/kg i.p. daily). Vitamin C did not interfere in leukocytes count, but when co-administered with cyclophosphamide, significant interaction was observed, intensifying the neutropenia caused by cyclophosphamide. Vitamin C did not influence body weight during treatment, but groups receiving cyclophosphamide had a significant weight loss from the third day of treatment until the end of experiment compared to the control group. Vitamin C supplementation intensified neutropenia induced by cyclophosphamide and did not prevent weight loss induced by cyclophosphamide in mice.

Keywords: Cyclophosphamide; Immunosuppression; Vitamin C; Weight loss.

RESUMEN

La neutropenia es uno de los efectos adversos causados por la administración de medicamentos de quimioterapia. El presente estudio tuvo como objetivo investigar el efecto de la administración de suplementos de vitamina C en un modelo de inmunosupresión inducida por ciclofosfamida en ratones. Se realizó la suplementación de vitamina C (50 mg/kg/día) administrada por vía intraperitoneal (i.p) por 7 días en ratones hembras swiss albinas adultas. Los ratones fueron divididos en cuatro grupos (n= 8 / grupo): Control (solamente agua destilada i.p) Ciclofosfamida (ciclofosfamida i.p. 150 mg/kg y 100 mg/kg en el día 1 y día 4 y

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agua destilada diariamente) Vitamin C (vitamina C 50 mg/kg i.p y agua destilada diariamente), Ciclofosfamida y Vit C (ciclofosfamida i.p. 150 y 100 mg/kg en el día 1 y día 4 y vitamina C 50 mg/kg i.p. diariamente). La vitamina C por sí no interfirió en los valores de leucocitos y tampoco influyó en el peso corporal durante el tratamiento, pero los grupos que recibieron ciclofosfamida tuvieron una pérdida de peso significativa desde el tercer día de tratamiento hasta el final del experimento en comparación con el grupo control. La suplementación de vitamina C intensificó la neutropenia inducida por ciclofosfamida y no evitó la pérdida de peso inducida por ciclofosfamida en ratones. Palabras clave: Ciclofosfamida; Inmunosupresión; Pérdida de peso; Vitamina C.

INTRODUCTION

Chemotherapy is one of the important methods used for the treatment of neoplastic diseases. Cyclophosphamide (CLF), an alkylating agent used to treat many types of neoplasia, is cytotoxic and has serious side effects, such as inhibition of acute transient bone marrow and myelosuppression^{1,2}. Leukopenia after chemotherapy is the common phenomenon of reduction in the number of leukocytes, which can lead to infections. One of the main reasons for concomitant infection is called neutropenia and can sometimes be associated with fever and more prone to serious infections³. The adverse effects caused by the administration of antineoplastic drugs usually develop after the first cycle of chemotherapy, as a consequence of the high doses administered. There is a cumulative effect that remains throughout the treatment, which can lead to weight loss and increased vulnerability to potential infections^{4,5,6}.

Several immunomodulatory nutrients have been studied as adjuvant in cancer treatment. Antioxidant supplementation, including vitamin C, can reduce the development of adverse effects associated with chemotherapy^{7,8,9}. This micronutrient has low cost, is easily accessible and safe¹⁰. In addition, supplementation of vitamin C in combination with cytotoxic agents in experimental models with CLF has shown therapeutic potential^{11,12,13}. Previous studies concluded that vitamin C supplementation is nephroprotective in mice exposed to cisplatin^{14,15}, indicating that this nutrient can be beneficial when administered in combination with chemotherapy agents. Moreover, pretreatment with vitamin C may effectively prevent radiation-induced gastrointestinal syndrome¹⁶. Vitamin C combined with radiotherapy, chemotherapy and hormone therapy reduced the side effects in antineoplastic treatment¹⁷, suggesting that supplementation can contribute to decreased oxidative chemical reactions¹⁸. In addition, high-dose vitamin C administration has been shown to modulate cytokine levels in patients with cancer and may be an adjuvant medicine combined with conventional chemotherapy drugs to induce cancer cell death¹⁹.

Evidence describing the effect of vitamin C on immune cells is widely described in the scientific literature. The immunomodulatory effect of vitamin C could improve the function of neutrophils²⁰, effect protection in sepsis settings²¹ as well as attenuate the toxicity effect on hematological parameters in total leukocyte²² and increase the phagocytic capacity of neutrophils²³.

The benefits of vitamin C supplementation associated with antineoplastic drugs toxicity is still controversial and its effects on neutropenia is not completely understood. This study aimed to evaluate the effect of vitamin C supplementation in a model of immunosuppression induced by CLF in mice.

MATERIALS AND METHODS

Animals

BALB/c female mice age 5-7 weeks and weighing 28.65 ± 2.99g were used. The experiment lasted 12 days (5 days for adaptation and 7 of treatment). Mice were maintained in a controlled environment (12:12 light/dark cycle) at a

temperature of 22 ± 2 °C. A standard pelleted diet and water were provided ad libitum. All animal experiments were conducted according to the Ethics Committee in Animal Research at the Federal University of Pelotas, N° 2415 (2015).

Immunosuppression was induced by CLF (Sigma-Aldrich®) injected intraperitoneally (i.p.) 150 mg/kg on day 1 of the induction and 100 mg/kg on day 4, as previously described by Zuluaga et al²⁴. The treatment was managed with 50 mg/kg of vitamin C (Synth®) dissolved in distilled water and administered i.p. daily, during six consecutive days.

Experimental design

Mice were divided into 4 groups (n= 8/group): I - CONTROL (distilled water i.p. daily); II - CLF (cyclophosphamide i.p. 150 mg/kg and 100 mg/kg on days 1 and 4, respectively, and distilled water daily); III - Vit C (50 mg/kg/day i.p. and distilled water daily); IV - Vit C + CLF (cyclophosphamide i.p. 150 mg/kg and 100 mg/kg on days 1 and 4, respectively, and vitamin C 50 mg/kg/day i.p. daily). All animals were submitted to two applications within the same condition. Daily body weight was measured on a digital scale (Mars® AD2000) for dose adjustment of vitamin C and weight control. On day 7, mice fasted for 6 hours before anesthesia was administered with ketamine 10% and xylazine 2% and euthanized.

Blood collection and hematological analysis

The blood samples were collected by cardiac puncture after anesthesia and immediately transferred into tubes containing EDTA. Total and differential white blood cell counts (Total Leukocyte– WBC, Monocytes, Lymphocytes and Neutrophils) were performed manually for each sample using the cell counter semi-automatic CC-530 (Celm). The samples were diluted in automatic diluter Celm Of-500 (Celm) and microscopic examination of Wright-stained smears with 100X objective²⁵.

Statistical analysis

Continuous variables were expressed as mean and standard error. The normality was verified for each variable to use the appropriate test. Two-way ANOVA and repeated measures two-way ANOVA were used to verify statistical differences and interactions between groups (effect of the CLF, Vit C and its interaction), with significance level of 5%. When interaction was observed, a t test was performed to compare individual groups. All analyses were carried out using GraphPadPrism 6® version 6.01.

RESULTS

Changes in body weight

On the first day (baseline), no differences were found between groups. No significant changes were observed in body weight until the third day, when CLF groups presented weight loss (CLF and Vit C + CLF). The control group, which received only water, Vit C group, that received just vitamin C, did not change body weight during the whole experiment. At the end

of the experiment, on day seven, CLF and Vit C + CLF groups had a significant weight loss when compared to baseline (19%).

It is important to highlight that on the third day, there was a significant weight loss in the CLF group (24%), which was partially recovered from the following day and that remain low until the end of experiment. Vit C + CLF group presented a slight but significant decrease (7%, $p < 0.05$) on the third day when compared to baseline, this loss persisted during the whole experimental period (Figure 1). On day 7, the weight of animals in Vit C + CLF group was not different from the CLF group ($p = 0.97$), but both groups had lower values compared to control group and to baseline values ($p = 0.02$; Figure 1).

Hematological parameters at the end of treatment

There was a significant difference in total leukocytes, lymphocytes and neutrophils count in groups receiving CLF ($p < 0.01$; factor cyclophosphamide). CLF reduced neutrophils by 97%, confirming the model (Figure 2a; 2c and 2d).

Vitamin C presented no effects on leukocytes, monocytes, lymphocytes count, but caused a significant decrease (35%) in neutrophil count (Figure 2d). However, this reduction does not characterize neutropenia, since it remained within normal limits. Co-administration of vitamin C and CLF presented a significant interaction ($p = 0.01$), as vitamin C intensified the neutropenia induced by CLF (Figure 2d), but did not affect values

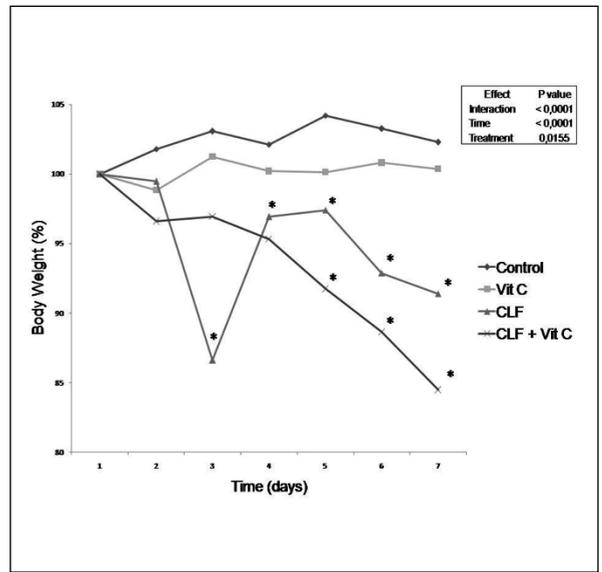


Figure 1. Weight during treatment. Percent change in body-weight during 7 days of the biological assay in adult Swiss albino females (n=8 per group). * $p < 0.05$ as compared to control (Repeat measures Two Way ANOVA).

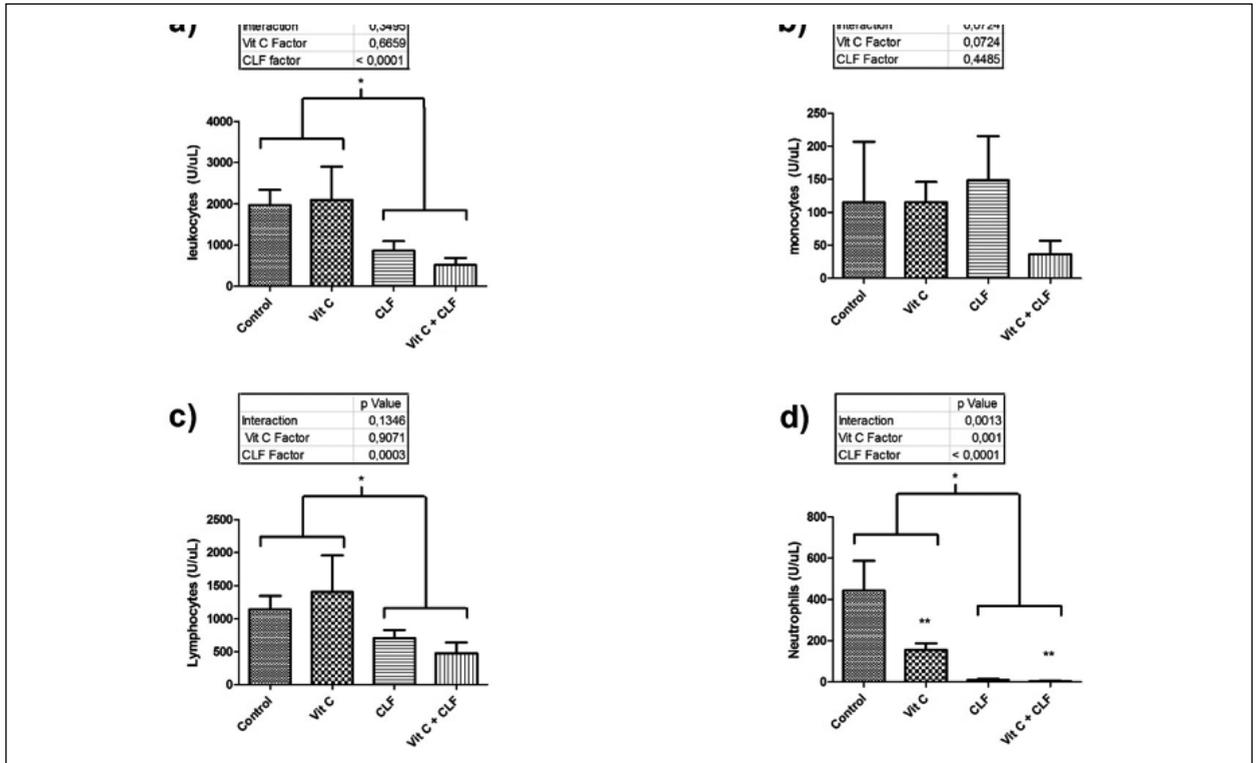


Figure 2. White blood cell parameters at the end of treatment. White blood cell count after 7 days of the biological assay, leukocytes (a), monocytes (b), lymphocytes (c), neutrophils (d) in adult Swiss albino females (n=8 per group). Results were expressed as mean \pm EP. ANOVA 2 way. *significant difference in Factor Cyclophosphamide, ** significant difference Factor Vitamin C.

of leukocytes, monocytes and lymphocytes (Figure 2a, 2b, 2c).

DISCUSSION

Vitamin C did not effective to prevent neutropenia but seemed to attenuate weight loss observed on day 3 after CLF. However, at the end of treatment, there was no difference between groups regarding body weight.

One adverse effect that can occur after the administration of CLF is neutropenia. Cyclophosphamide-induced bone marrow suppression reduces the number of white blood cell, red blood cell and platelets²⁶. Neutropenia is a condition in which the number of neutrophils in the blood stream is decreased, defined as a neutrophil count lower than 1.5g/L, with categorization as mild, moderate, or severe when the count is 1.5-1g/L, 1-0.5g/L, or <0.5g/L, respectively²⁷. Neutrophils are the most abundant white blood cell in the blood and play a critical role in preventing infections as part of the innate immune system. Neutrophils are the first cells in the line of defense and are responsible for phagocytosis, therefore, they would be the first to show the effects caused by CLF²⁸.

In the current study, we observed 97% reduction in the number of neutrophils after CLF administration. Vitamin C, while being immunomodulatory nutrient, did not prevent the depletion effect of CLF. Similarly, a study using CLF reported that the number of leukocytes began to decline in the first day after the induction, reaching minimum values on day 3 and increased gradually from day 7²⁹. These results are in agreement with our research, since at day 7 after the induction model both leukocytes and lymphocytes, were significantly lower (56% and 38%, respectively) than the control group. These findings indicate that the immunosuppression model was effective. However, the intensity of neutropenia may change according to the dose and administration time of the chemotherapeutic agent^{30,31}.

Vitamin C caused a reduction in neutrophils count within the normal limits. When vitamin C was co-administered with CLF, there was intensification of the CLF-induced neutropenia. Another study showed that oral supplementation of vitamin C in mice exposed to a chemotherapeutic agent was not able to prevent a decrease in total leukocytes, lymphocytes and neutrophils counts³². However, long-term high-dose intake of vitamin C is effective in the maintenance of immune cells³³.

Vitamin C it is a highly effective antioxidant, due to its ability to readily donate electrons. Vitamin C can also scavenge numerous reactive oxidants and can modulate immune function through redox-sensitive cell signaling pathways or directly by protecting important cell structural components³⁴. In addition, studies evaluating different doses of natural immunomodulatory antioxidants (e.g., glucan, resveratrol and vitamin C)³⁵ and vitamin E, C and K3 vitamin cocktail could minimize the effects of chemotherapeutic agent³⁶. Therefore, the effect found with vitamin C supplementation alone could be different from when co-administered with other vitamins, which may help to restore normal blood cell counts and function.

Recently, our group showed a possible mechanism involved in CLF-mediated damage caused by imbalance of the redox state through the reduction of glutathione (GSH) and lipid peroxidation in hepatic tissue of mice submitted to the same experimental model of neutropenia. We found that vitamin C supplementation was not effective in protecting against oxidative stress, but prevented the decrease in endogenous GSH³⁷.

Explanation likely why vitamin C supplementation did not prevent neutropenia may be the fact that CLF produces free radicals and may decrease the antioxidant action of vitamin C. Thus, it is assumed that oxidative stress may be mediating the effects of vitamin C and increasing the toxicity of the chemotherapy agent³⁸.

The use of CLF in mice has been related to weight loss^{26,39}. A previous study showed that CLF reduced the weight of mice by approximately 7% after 30 days of treatment²⁹. This difference can be explained by the different intervals that mice were weighed. Thus, weight may have been recovered after reaching the nadir, in which mice were more debilitated, as the lower dose used may also have influenced the lower weight loss throughout the treatment.

In the present study, CLF resulted in a body weight reduction of 19% and vitamin C supplementation did not attenuate weight loss induced by cyclophosphamide. However, weight loss was more gradual than the acute effect observed in the group that did not receive vitamin C. This significant weight loss may have occurred due to the side effects of the drug (e.g., diarrhea, nausea, and/or inappetence) as well the immunosuppressive state that may result in reduced feed intake and body weight loss as observed in humans⁴⁰, but these side effects were not evaluated in this study.

Additionally, significant weight loss was described in a study conducted in mice exposed to CLF and radiation therapy, with 30% weight loss after 10 days of treatment³¹. The higher weight loss may be due to the combination of radiation and chemotherapy effects. The delay in weight gain in rats exposed to CLF found in other studies^{26,41}, as well as in the present study, may be related to the side effects of CLF, such as mucositis and dehydration.

Vitamin C as an immunomodulatory nutrient with a strong potential against gastrointestinal damage induced by antineoplastic drugs⁴² and is also able to contribute to the reduction of symptom severity^{43,44}, which could explain gradual weight loss throughout the study.

CONCLUSION

Vitamin C supplementation (50 mg/kg) for 7 consecutive days did not prevent neutropenia, but rather intensified neutropenia when co-administrated with cyclophosphamide in mice. Vitamin C did not prevent weight loss induced by CLF in female mice, but attenuated acute weight loss after the application of chemotherapy. More studies should be performed focusing on vitamin C supplementation.

Abbreviations: CLF: cyclophosphamide; Vit C: vitamin C; i.p.: intraperitoneally.

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