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The protective effects of nutritional antioxidant therapy on Ehrlich solid tumor-bearing mice depend on the type of antioxidant therapy chosen: histology, genotoxicity and hematology evaluations☆

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Abstract

Strong evidence indicates that reactive oxygen species (ROS) play an important role in the initiation as well as the promotion phase of carcinogenesis. Studies support the role of ROS in cancer, in part, by showing that dietary antioxidants act as cancer-preventive agents. Although results are promising, the research on this topic is still controversial. Thus, the aim of this study was to investigate whether vitamins C, E and pequi oil can, individually, provide prevention and/or be used afterward as an adjuvant in cancer therapy. Ehrlich solid tumor-bearing mice received antioxidant as follows: before tumor inoculation, before and after tumor inoculation (continuous administration), and after tumor inoculation; morphometric analyses of tumor, genotoxicity and hematology were then carried out. Antioxidant administrations before tumor inoculation effectively inhibited its growth in the three experimental protocols, but administrations after the tumor's appearance accelerated tumor growth and favored metastases. Continuous administration of pequi oil inhibited the tumor's growth, while the same protocol with vitamins E and C accelerated it, favoring metastasis and increasing oxidative stress on erythrocytes. Except for continuous administration with vitamin E, the development of ascites tumor metastases was linked with increased inflammation. Results suggest that the efficiency and applicability of antioxidants in the medical clinic can depend not only on the nature of the antioxidant, the type and stage of cancer being treated and the prevailing oxygen partial pressure in the tissues, but also on the type of antioxidant therapy chosen.

Keywords: Solid Ehrlich tumor; Antioxidant therapy; Oxidative stress; Comet assay; hemogram; Tumor morphometry

1. Introduction

Strong evidence indicates that reactive oxygen species (ROS) play an important role in the initiation as well as the promotion phase of carcinogenesis [1,2]. Experimental studies support the role of ROS in cancer, in part, by showing that dietary antioxidants act as cancer-preventive agents. These include vitamins C and E, β-carotene and other phytochemicals, as well as endogenous antioxidants (e.g., glutathione) that neutralize or trap ROS [1].

Although studies involving nutritional antioxidant therapy in cancer treatment are promising, research on this topic is still scarce and controversial [3–6]. Studies indicate that some antioxidant nutrients minimize the toxic effects produced by antineoplastic drugs, interfering positively in response to the treatment [3,7]. On the other hand, radiation therapy and some chemotherapy drugs generate ROS, and antioxidants may prevent cancer cells from being killed by ROS. Thus, many oncologists have turned against antioxidants during conventional cancer therapy [6]. Additionally, there have been no adequate randomized controlled trials to evidence the efficiency and applicability of antioxidants during chemotherapy or radiotherapy [8].

Although it occurs independently of the chosen treatment, antioxidant protection of normal cells takes place in all treatments, since antioxidants protect normal tissues from the toxic effects of ROS [6]. However, there are some aspects that need to be better investigated before antioxidant intervention. Potential effects on treatment effectiveness depend on the nature of the antioxidant (and its dose), the chemotherapy drugs being used, the type and stage of cancer being treated [1,6,7] and the prevailing oxygen partial pressure (Po_2) in the tissues [1,9]. Therefore, all these points must be taken into consideration for a nutritional antioxidant therapy. Carotenoids, for example, are an effective antioxidant under low PO_2 conditions [1,9], whereas under high PO_2, they are less efficient and may even act as pro-oxidants due to auto-oxidation [1]. On the other hand, vitamin E (α-tocopherol) is an efficient antioxidant for cells submitted to high

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Table 1
Relative composition of pequi (*C. brasiliense* Camb.) pulp oil

Fatty acids [13] [a] (%)						Carotenoids [14–17] (mg/100 g of pequi fruit pulp)		
Saturated		Monounsaturated		Polyunsaturated		Provitamin A	Lycopene	Total
Palmitic	41.78	Oleic	54.28	Linoleic	1.36	6.26−11.5	1.12−2.08	6.75−28.66
Stearic	1.28	Palmitoleic	0.67	Linolenic	0.51			
Araquidic	0.12							

[a] Present study.

PO_2, such as those of the lungs [1]. As a result, studies that evaluate the effect of different antioxidants in the prevention and treatment of one type of tumor can contribute to greater understanding of the potential effect of these substances in treatment effectiveness.

Thus, the aim of this study was to evaluate the effect of different antioxidant interventions on one type of tumor and to investigate whether antioxidant supplements can, individually, provide protection and/or be used afterward as an adjuvant in cancer therapy. For this, Ehrlich solid tumor was chosen as the experimental model. This tumor is a neoplasm of epithelial malignant origin, corresponding to murine mammary adenocarcinoma. It is easily cultivated and transferred *in vivo* and is widely used for experimental evaluations [10]. The antioxidant supplements chosen for tests were vitamins C (ascorbic acid) and E (α-tocopherol) and pequi fruit pulp oil, a carotenoid-rich oil extracted from pequi (*Caryocar brasiliense* Camb.), a typical fruit of the Brazilian Cerrado used in folk medicine to treat several diseases [11], including in control of tumors [12].

2. Materials and methods

2.1. Chemicals

Ketamine and xylazine were obtained as chlorhydrate. Ketamine sold as Dopalen 100 mg/ml was obtained from Ceva Animal Health Ltd (São Paulo, Brazil) and xylazine (Coopazine 20 mg/ml) came from Coopers (São Paulo, Brazil). Vitamin C (ascorbic acid), sold as Citroplex 200 mg/ml, was obtained from Neo Química Laboratory (Goiás, Brazil) and vitamin E (α-tocopherol), 400 mg per capsule, was obtained as DL-α-tocopheryl acetate from Sandoz Pharmaceutical Industry (Paraná, Brazil).

2.2. Plant material

Pequi fruit was obtained *in natura* from the local markets of Brasília/DF (Brazil) and surrounding areas. The internal mesocarp was peeled or grated to obtain the pulp, which was packed in a covered pot and frozen at −86°C. Pequi pulp oil was extracted by cold maceration using chloroform as a solvent. The extract was subjected to evaporation under reduced pressure and dried at high vacuum for complete solvent removal. The relative composition of the pequi fruit pulp oil is shown in Table 1.

2.3. Ehrlich tumor

The Ehrlich ascitic tumor, derived from a spontaneous murine mammary adenocarcinoma, was maintained in ascitic form by passages in Swiss mice by weekly intraperitoneal transplantation of 10^6 tumor cells [18]. The ascitic fluid was collected by intraperitoneal puncture using a sterile insulin syringe. Ascitic tumor cell counts were done in a Neubauer hemocytometer. The cells were found to be more than 99% viable by the trypan blue dye exclusion method.

2.4. Animals and experimental design

Female Swiss albino mice, 8 to 12 weeks old, weighing 28.9±2.4 g, obtained from the animal facility of the University of Brasilia (Brazil), were housed in plastic cages (six per cage) at room temperature (20°C±2°C) in a 12-h light/dark cycle with lights on at 6 a.m. and free access to food and water.

The animals were anesthetized by intraperitoneal administration of ketamine (80 mg/kg) and xylazine (10 mg/kg), both in the same syringe, in a final dose of 0.1 ml/30 g. A volume of 0.04 ml (5.5×10^4 viable cells) of Ehrlich ascitic tumor cell suspension was injected subcutaneously in the upper region of the head (line formed between the two ears, when these are pulled slightly upward) for the solid-form implantation. Daily dose of the antioxidants was calculated using the dose translation formula developed by Reagan-Shaw et al. [19], obeying the maximum daily dose of provitamin A carotenoids (25 mg), vitamin E (1200 IU) and vitamin C (1 g) for adult humans recommended by the National Agency for Sanitary Surveillance. Mice (n=6 per group) were treated with pequi oil 30 mg/animal/day or vitamin C 4 mg/animal/day or vitamin E 20 mg/animal/day administered orally by gavage, as follows: (1) antioxidant, daily for 10 days, followed by tumor implantation (administration "before"); (2) antioxidant, daily for 10 days, followed by tumor implantation and continued daily antioxidant administration for 15 more days (administration "continuous"); (3) tumor implantation followed by antioxidant, daily for 15 days (administration "after"). Negative controls (n=6 per group) received filtered water, pequi oil, vitamin E or vitamin C, and no tumor implantation took place. In the positive control (n=6), tumor was implanted and no antioxidant was administered. Euthanasia was carried out 15 days after tumor implantation by cervical dislocation.

All procedures described were reviewed and approved by the institutional Ethics Committee for Animal Research (Institute of Biological Science, University of Brasília), number 107748/2009.

2.5. Procedures and measurements

Before euthanasia, the animals were anesthetized with a mixture of xylazine and ketamine according to the method described above. Blood samples (1 ml) collected by cardiac puncture, using an insulin syringe containing EDTA as anticoagulant, were used to carry out comet assay and hemogram. The latter test was processed in a multiple automated hematology analyzer for veterinary use, Sysmex pocH-100iV Diff (Curitiba/Paraná, Brazil) calibrated for mice. Euthanasia of the animals was carried out by cervical dislocation. Tumors were removed; width and length were measured using a digital pachymeter; tumor volume was calculated according to Yanase et al. [20]. Afterward, tumors were fixed with 10% formalin for 24 h, transferred to 70% ethanol, included in paraffin using a automatic tissue processor (OMA DM-40, São Paulo, Brazil), cut to 5-μm thickness in a Leica RM2235 manual microtome (Leica Microsystems, Nussloch, Germany) and stained with hematoxylin–eosin for histological analyses (light microscopy). Computer software for image analysis (Image Pro Plus, Media Cybernetics) was utilized for measurements of tumor necrosis area.

Table 2
Animal body weight and results of comet assay of Ehrlich solid tumor-bearing mice before and after antioxidant administration

		Treatment	Group	Body weight (g)	Comet assay (AU)
Without tumor		Without treatment	1	26.57±0.98	126.98±2.37
		Pequi	2	28.84±1.09	106.52±2.64 **a
		Vitamin E	3	29.49±0.69	107.83±5.18 *a
		Vitamin C	4	28.38±1.09	104.78±5.39 **a
With tumor		Without treatment	5	29.01±0.93	127.46±4.26 **b,d, *c
	Pequi	Before	6	28.04±0.63	116.05±3.10
		Continuous	7	28.15±1.49	118.10±2.14
		After	8	28.58±0.62	130.58±4.02 **b
	Vitamin E	Before	9	30.32±0.66	119.13±2.92
		Continuous	10	29.87±0.46	117.17±1.93
		After	11	29.57±0.69	131.68±2.35 **c
	Vitamin C	Before	12	29.15±0.92	90.09±6.02 **a,e,f,h
		Continuous	13	29.31±0.5	113.84±2.12 **j
		After	14	29.46±0.27	109.78±2.49 **g,i, *j
			P	.187	.000

P values were generated by ANOVA.
Superscript letters indicate significant differences detected by the Bonferroni test: [a]compared with Group 1; [b]compared with Group 2; [c]compared with Group 3; [d]compared with Group 4; [e]compared with Group 5; [f]compared with Group 6; [g]compared with Group 8; [h]compared with Group 9; [i]compared with Group 11; [j]compared with Group 12.
* $P<.05$, significant difference.
** $P<.01$, highly significant difference.

2.6. Single-cell gel electrophoresis (comet assay)

The comet assay (alkali method) was performed according to the method developed by Singh et al. [21], with a few modifications. Briefly, 20 μl total blood of each sample was mixed with 120 μl of 0.5% low-melting-point agarose in phosphate-buffered saline (Gibco BRL, Grand Island, NY) at 37°C and pipetted onto eight microscope slides precoated with a layer of 1.5% normal-melting-point agarose prepared in phosphate-buffered saline. Slides were then immersed in a freshly prepared cold (4°C) lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0–10.5, 1% lauroyl sarcosine, with 1% Triton X-100 and 10% dimethyl sulfoxide added fresh) for 1 h at 4°C. After lysis, slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH>13.0) and left in the solution for 40 min at 4°C. Electrophoresis was conducted at 4°C for 30 min at 25 V (0.8 V/cm) and 300 mA. Once completed, slides were washed three times with neutralizing solution (0.4 M Tris, pH 7.5), stained with EtBr (20 μg/ml) and analyzed with a Zeiss Axioskop 2 fluorescence microscope (filter 510–560 nm, barrier filter 590 nm) with a total magnification of ×400. One hundred comets on each slide were scored visually by a trained professional as belonging to one of the five classes proposed by Collins et al. [22], and the DNA damage was calculated according to Jaloszynski et al. [23] (giving a maximum possible score of 400, corresponding to 100 cells in Class 4). Each treatment was independently repeated twice. Moreover, in all cases, preliminary experiments were performed to single out the best experimental conditions.

2.7. Statistical analysis

Statistical analysis was carried out using SPSS version 15.0 (Statistical Package for the Social Sciences, Chicago, IL). Data were expressed as mean±S.E.M. and values of $P<.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro–Wilk. For animals' body weight, the differences among groups were checked by ANOVA, while differences in other variables were investigated by ANOVA or Kruskal–Wallis test (when the "data were not normally distributed). For significant ANOVA results, Bonferroni's post hoc test was chosen to carry out two-to-two comparisons between the treatments. For significant Kruskal–Wallis results, Mann–Whitney *U* test was performed to verify differences between the treatments (two-to-two comparisons). The following groups were statistically compared: (1) negative filtered water control×all other groups; (2) negative antioxidant controls among themselves; (3) negative pequi oil control×pequi oil treatments; (4) negative vitamin E control×vitamin E treatments; (5) negative vitamin C control×vitamin C treatments; (5) positive control×all treatments after tumor

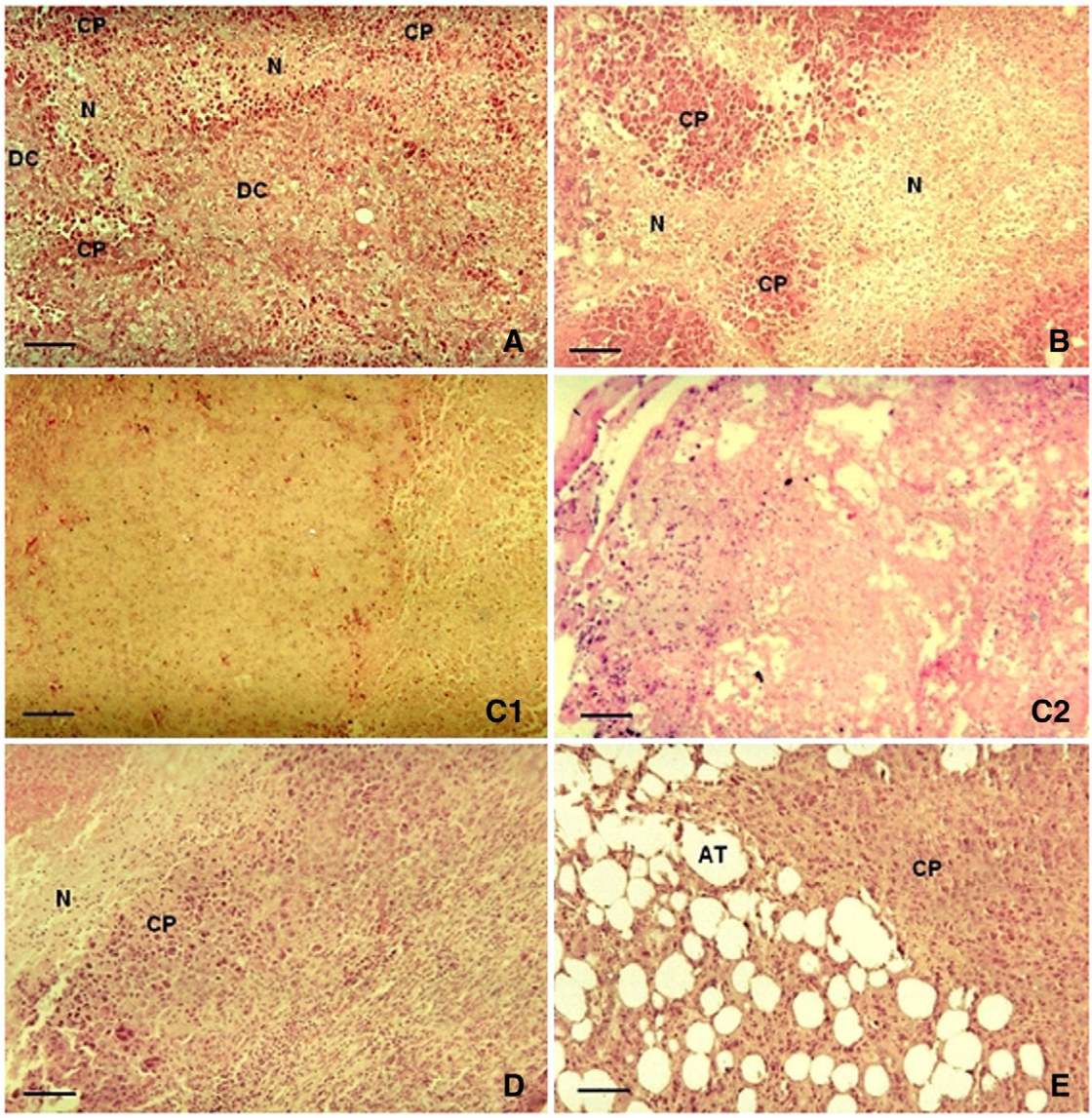

Fig. 1. Ehrlich solid tumor histology before and after antioxidant administration (total magnification ×100). (A and B) Positive control: small, higher chromatophilic tumor cells of variable shape represent cell proliferation (CP) regions surrounding areas of necrosis (N) and differentiated cells (DC), represented for larger and less chromatophilic tumor. (C) Preventive administrations (before tumor inoculation) with (C1) pequi oil and (C2) vitamin E: there were extensive necrosis regions containing pycnotic nuclei (better visualized in C2). (D) Vitamin E treatment (after tumor inoculation): cells lying deeply within solid tumors were smaller than their more peripherally placed counterparts and necrosis regions were internally located. (E) Vitamin C treatment: tumor cells advancing through adipose tissues (AT). N, necrosis; CP, cell proliferation; DC, differentiated cells.

inoculation; (6) "before" treatments among themselves; (7) "continuous" treatments among themselves; and (8) "after" treatments among themselves.

3. Results

There were no significant differences in animals' body weight among the analyzed groups (Table 2).

3.1. Comet assay

Antioxidant supplementation given to healthy animals (without tumor) significantly reduced DNA damages in comparison to the negative filtered water (Group 1) and positive (Group 5) controls (Table 2). For the inoculated animals, the greatest reduction in DNA damage was observed with vitamin C before tumor inoculation (Group 12). Although the same protocol with pequi oil (Group 6) and vitamin E (Group 9) also led to a reduction in DNA damage in comparison to the negative filtered water and positive controls, these decreases were not significant. The same reductions occurred with the continuous administration of pequi oil (Group 7), vitamin E (Group 10), vitamin C (Group 13) and with vitamin C administration after tumor inoculation (Group 14). Administrations of pequi oil (Group 8) and vitamin E (Group 11) after tumor inoculation produced significant increases in DNA damage in comparison to their respective antioxidant controls. Among the "before" treatments, vitamin C presented a significant reduction in DNA damage compared with that presented by pequi oil. This treatment with vitamin C also presented significantly lower DNA damage than for the other protocols with the same vitamin.

3.2. Tumor histology and morphometric analyses of necrosis area

The peripheral regions of positive control slides of solid Ehrlich tumors were typically composed of areas of cell proliferation surrounding areas of differentiated cells; necrosis regions were generally found more deeply (Fig. 1A). Large solid tumors had extensive regions of internal necrosis, while in small tumors these regions were minor. Islands containing regions of cell proliferation and pycnotic nuclei were also observed (Fig. 1B).

Antioxidant administrations before tumor inoculation (Groups 6, 9 and 12) presented spread islands of differentiated cells immersed in extensive necrosis regions. Cell proliferation areas were scarce or absent; the tumors were dying (Fig. 1C). Morphometric measurements of the necrosis area showed a significant increase in necrosis for the treatment with pequi oil before tumor inoculation, although nonsignificant increases in this area were also observed with vitamin E (Table 3).

Continuous administrations with vitamins E and C (Groups 10 and 13) presented similar histology to the positive control slides, but the dimensions of the necrosis area were significantly lower in the treatment with vitamin C. However, the same administration with pequi (Group 7) supplied results that were similar to those observed for pequi oil administration before tumor implantation; this similarity was found in both histology and morphometric measurements of the necrosis area (Table 3). All the protocols of tumor implantation followed by antioxidant administration presented similar histology to those of the positive controls (Fig. 1D), but morphometric analyses showed a significant reduction in the necrosis area for the treatment with vitamin C (Table 3). Continuous administration with vitamins E (Group 10) and C (Group 13) and vitamin C administered after tumor inoculation (Group 14) also presented invading tumor cells advancing through adipose tissues (Fig. 1E).

3.3. Tumor dimensions and metastasis

Antioxidant administrations before tumor inoculation significantly reduced tumor volume when compared with the positive control (Table 3), and among this group, it was vitamin C that produced the greatest reduction. The same tumor reduction result was also observed for continuous administration with pequi oil (Group 7). For vitamins E and C, continuous administration resulted in metastasis (Groups 10 and 13), which was also observed in all protocols of tumor implantation followed by antioxidant administration (Groups 8, 11 and 14).

3.4. Hematology

In the erythrogram (Table 4), red blood cells (RBCs) values of the negative filtered water control were not significantly different from those of the antioxidant control groups (Groups 2, 3 and 4) and from the group treated with pequi oil after tumor inoculation (Group 8). Hemoglobin (HGB) values of the pequi oil and vitamin E controls did not differ significantly from the negative filtered water control, while vitamin C control and all other treatments presented a significant

Table 3
Morphometric analyses and metastasis of Ehrlich solid tumor inoculated in mice before and after antioxidant administration

		Treatment	Group	Necrosis area (nm^2)	Width (mm)	Length (mm)	Thickness (mm)	Volume (mm^3)	Metastasis
With tumor		Without treatment	5	346.01±127.78	8.50±1.23	11.35±1.68	4.92±0.95	540.15±238.64	—
	Pequi	Before	6	851.66±95.32 ^{*a}	5.68±0.78	7.16±0.86	3.20±0.32	153.71±69.71 ^{*a}	—
		Continuous	7	663.69±170.78	4.56±0.66	7.12±1.22	1.99±0.32 ^{**a,*b}	101.34±40.83 ^{*a}	—
		After	8	230.93±195.73 ^{*b}	9.79±1.78	17.26±3.32	5.85±1.60 ^{**c}	1086.60±470.85 ^{**c}	S (head) and A (peritoneum)
	Vitamin E	Before	9	639.62±225.51	4.28±0.59	6.36±0.62	2.51±0.48	69.22±22.41 ^{*a}	—
		Continuous	10	221.64±117.7	7.10±0.63	9.06±1.05	2.37±0.55 ^{*a}	242.57±61.45 ^{**e}	S (neck to cervical region) and A (upper back to thoracic region)
		After	11	92.04±22.80	8.67±1.48	11.86±2.08	2.37±0.55 ^{*a}	643.57±309.42 ^{*e}	S (head)
	Vitamin C	Before	12	230.14±32.42 ^{**b}	4.19±0.23	5.58±0.49	1.85±0.39 ^{*a,b}	50.85±8.09 ^{**a}	—
		Continuous	13	22.08±7.87 ^{*a,c,f}	9.22±1.81	14.40±3.57	2.57±0.44 ^{*a}	1897.84±1406.05 ^{*c}	S (head, neck and lower back) and A (upper back)
		After	14	24.87±8.43 ^{*a,f}	10.61±1.92	16.95±4.51	2.03±0.33 ^{*a,**d}	1603.34±822.30 ^{**f}	S (head, neck and upper back) and A (head, neck and upper back)
			P	.000	.013	.026	.008	.001	

S, solid tumor; A, ascites tumor.
Tumor volume=0.5×(length×width^2) [20].
P values for width and length were generated by ANOVA, while *P* values for necrosis area, thickness and tumor volume were generated by the Kruskal–Wallis test.
Superscript letters indicate significant differences detected by the Bonferroni or the Mann–Whitney *U* tests in the two-to-two comparisons: [a]compared with Group 5; [b]compared with Group 6; [c]compared with Group 7; [d]compared with Group 8; [e]compared with Group 9; [f]compared with Group 12.
* *P*<.05, significant difference.
** *P*<.01, highly significant difference.

Table 4
Erythrogram of Ehrlich solid tumor-bearing mice before and after antioxidant administration

		Treatment	Group	RBC ($\times 10^6$/μl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)
Without tumor		Without treatment	1	8.57±0.10	12.75±0.18	31.75±0.43	37.05±0.42	14.87±0.14	40.18±0.44	13.95±0.44
		Pequi	2	8.80±0.12	13.05±0.22	32.45±0.42	36.87±0.15	14.85±0.09	40.20±0.31	14.40±0.38
		Vitamin E	3	8.66±0.12	12.73±0.20	31.97±0.52	36.92±0.29	14.70±0.14	39.85±0.13	14.07±0.25
		Vitamin C	4	8.23±0.16	11.78±0.27 *a,c, **b	29.57±0.72	35.92±0.30	14.32±0.11	39.88±0.29	15.60±0.26
With tumor		Without treatment	5	7.86±0.24 *a,c, **b	11.30±0.34 **a,b,c	28.70±0.99	36.50±0.27	14.37±0.12	39.43±0.47	15.77±0.47
	Pequi	Before	6	7.81±0.10 **a,b	11.30±0.18 **a,b	29.19±0.52	37.39±0.42	14.46±0.16	38.71±0.28	16.50±0.5
		Continuous	7	8.12±0.13 *a, **b	11.67±0.20 **a,b	29.93±0.52	36.86±0.27	14.39±0.13	39.01±0.38	16.37±0.45
		After	8	8.10±0.25 *b	11.42±0.29 **a,b	29.20±0.82	36.08±0.26	14.12±0.18	39.13±0.33	15.82±0.75
	Vitamin E	Before	9	7.93±0.14 **a,c	11.17±0.17 **a,c	28.73±0.35	36.27±0.31	14.08±0.07 *a	38.85±0.22	15.58±0.58
		Continuous	10	6.64±0.49 **a,c,f, *e,h	9.40±0.68 **a,c,f,h, *e	24.35±1.89 **a,c,f, *e,h	36.58±0.31	14.18±0.15	38.70±0.44	18.43±0.89 **a,c
		After	11	7.35±0.12 **a,c, *g,h	10.52±0.19 **a,c, *g,h	27.25±0.34 *a, **c	37.08±0.31	14.32±0.11	38.57±0.27	16.87±0.41 *a
	Vitamin C	Before	12	8.06±0.14 *a	11.76±0.18 *a	29.26±0.40	36.34±0.47	14.62±0.09	40.20±0.44	14.26±0.44
		Continuous	13	7.41±0.16 **a,d, *f,j	10.58±0.27 **a, *d,f,j	27.00±0.68 **a	36.41±0.36	14.25±0.17	39.19±0.29	16.74±0.50 *a
		After	14	7.54±0.16 **a, *d,i	10.65±0.28 **a, *d,j	27.37±0.75 *a	36.27±0.31	14.13±0.15	38.92±0.25	16.58±0.67
			P	.000	.000	.000	.132	.001	.002	.000

P values of RBC and HGB were generated by the Kruskal–Wallis test, while *P* values of the other parameters were generated by ANOVA.
Superscript letters indicate significant differences detected by the Bonferroni or the Mann–Whitney *U* tests in the two-to-two comparisons: [a]compared with Group 1; [b]compared with Group 2; [c]compared with Group 3; [d]compared with Group 4; [e]compared with Group 5; [f]compared with Group 7; [g]compared with Group 8; [h]compared with Group 9; [i]compared with Group 11; [j]compared with Group 12.
* *P*<.05, significant difference.
** *P*<.01, highly significant difference.

reduction in these values. However, mean corpuscular hemoglobin (MCH) values for the negative filtered water control only differed from the group that received vitamin E administered before tumor inoculation (Group 9, *P*=.002); for this group, the values decreased. Although ANOVA showed significant differences in mean corpuscular hemoglobin concentration (MCHC) values among the groups, there were no differences between two specific groups, nor were there any significant differences for mean corpuscular volume (MCV) values. For red cell distribution width (RDW), values of continuous administration with vitamins E (Group 10, *P*=.000) and C (Group 13, *P*=.010) and treatment with vitamin E after tumor implantation (Group 11, *P*=.040) increased significantly in comparison to the negative control.

In the leukogram (Table 5), there was a significant increase in values for neutrophils+monocytes in the positive control (Group 5, *P*=.041), vitamin C control (Group 4, *P*=.009), vitamin E before (Group 9, *P*=.009) and after (Group 11, *P*=.002) tumor inoculation, continuous administration with vitamin C (Group 13, *P*=.001) and vitamin C after tumor implantation (Group 14, *P*=.002), when compared with the negative control. There were no significant differences for white blood cells (WBCs), lymphocytes and eosinophils.

Results of plateletgram (Table 6) presented increased platelets (PLT) for vitamin C control (*P*=.015) and for the three antioxidant treatments after tumor implantation (*P*=.002 for pequi oil; *P*=.009 for vitamin E; *P*=.009 for vitamin C) in the comparisons with negative filtered water control. Vitamin E supplementation of healthy animals (Group 3) significantly reduced P-LCR (platelet large cell ratio) values (*P*=.041), while for the pequi oil administered before tumor inoculation (Group 6), these values increased (*P*=.015) when they were compared with the negative filtered water control. No significant differences were observed in platelet distribution width (PDW) values.

Table 5
Leukogram of Ehrlich solid tumor-bearing mice before and after antioxidant administration

		Treatment	Group	WBC ($\times 10^3$/μl)	Lymphocytes ($\times 10^3$/μl)	Neutrophils+monocytes ($\times 10^3$/μl)	Eosinophils ($\times 10^3$/μl)
Without tumor		Without treatment	1	3.58±0.34	2.40±0.28	1.13±0.10	0.05±0.05
		Pequi	2	4.15±0.54	2.87±0.50	1.27±0.18	0.02±0.02
		Vitamin E	3	3.93±0.33	2.42±0.23	1.47±0.23	0.05±0.02
		Vitamin C	4	6.45±0.75	4.43±0.54	2.00±0.26 *a,b	0.02±0.02
With tumor		Without treatment	5	5.88±0.66	3.22±0.38	2.62±0.68 *a	0.05±0.03
	Pequi	Before	6	5.67±1.13	3.46±0.61	2.17±0.78	0.04±0.02
		Continuous	7	4.29±0.66	2.87±0.41	1.39±0.31	0.03±0.02
		After	8	7.20±1.53	2.80±0.37	4.32±1.41 **a, *b	0.08±0.07
	Vitamin E	Before	9	6.50±0.61	4.00±0.26	2.47±0.71 *a	0.03±0.02
		Continuous	10	7.38±1.84	2.65±0.48	4.67±1.48 *e	0.07±0.03
		After	11	6.63±0.38	2.95±0.27	3.68±0.48 **a, *c	0.00±0.00
	Vitamin C	Before	12	5.98±0.28	4.66±0.33	1.32±0.11 *d,f	0.00±0.00
		Continuous	13	5.78±0.4	2.86±0.28	2.84±0.40 **a, *e,g	0.08±0.03
		After	14	7.65±0.61	3.70±0.64	3.93±0.63 **a,g, *d	0.02±0.02
			P	.008	.004	.000	.554

P values of WBC and lymphocytes were generated by ANOVA, while *P* values of neutrophils+monocytes and eosinophils were generated by the Kruskal–Wallis test.
The superscript letters indicate significant differences detected by the Bonferroni or the Mann–Whitney *U* tests in the two-to-two comparisons: [a]compared with Group 1; [b]compared with Group 2; [c]compared with Group 3; [d]compared with Group 4; [e]compared with Group 7; [f]compared with Group 9; [g]compared with Group 12.
* *P*<.05, significant difference.
** *P*<.01, highly significant difference.

Table 6
Plateletgram of Ehrlich solid tumor-bearing mice before and after antioxidant administration

		Treatment	Group	PLT ($\times 10^3/\mu l$)	MPV (fl)	P-LCR (%)	PDW (fl)
Without tumor		Without treatment	1	1142.83±73.77	6.63±0.06	8.38±0.53	6.78±0.07
		Pequi	2	1223.33±102.51	6.57±0.19	8.65±1.05	6.78±0.10
		Vitamin E	3	1257.67±27.90	6.27±0.08	7.07±0.24 *a	6.62±0.05
		Vitamin C	4	1518.00±85.49 *a,c	6.68±0.05	7.87±0.23	6.98±0.15
With tumor		Without treatment	5	1337.83±68.05	6.97±0.20	10.03±1.70 *c	6.85±0.15
	Pequi	Before	6	983.57±61.79 **e	7.23±0.12	11.03±0.54 *a	7.28±0.13
		Continuous	7	1343.14±95.50 *f	6.97±0.13	9.41±0.99	7.13±0.11
		After	8	1617.00±69.96 **a,f, *b,e	6.57±0.14	8.03±0.85 *f	6.77±0.15
	Vitamin E	Before	9	1317.50±96.40 **f	7.03±0.10 *c	9.93±0.64 **c	7.07±0.14
		Continuous	10	1311.67±208.95	7.02±0.10 *c	9.52±0.49 **c	6.98±0.23
		After	11	1568.50±68.93 *a,c,e	6.53±0.09	6.58±0.58 *e,g,h	6.75±0.10
	Vitamin C	Before	12	1119.20±43.56 *d,e	6.56±0.15	7.22±0.83 **f	6.74±0.15
		Continuous	13	1400.58±170.63	6.98±0.15	10.06±1.19	7.03±0.11
		After	14	1646.00±135.54 *a,i	6.75±0.11	8.05±0.59	6.95±0.15
			P	.002	.000	.003	.051

Platelet indices: PLT, mean platelet volume (MPV), P-LCR and PDW.
P values of PLT and P-LCR were generated by the Kruskal–Wallis test, while *P* values of MPV and PDW were generated by ANOVA.
The superscript letters indicate significant differences detected by the Bonferroni or the Mann–Whitney *U* tests in the two-to-two comparisons: [a]compared with Group 1; [b]compared with Group 2; [c]compared with Group 3; [d]compared with Group 4; [e]compared with Group 5; [f]compared with Group 6; [g]compared with Group 9; [h]compared with Group 10; [i]compared with Group 12.
* *P*<.05, significant difference.
** *P*<.01, highly significant difference.

4. Discussion

Many cancer-chemopreventive agents possess antioxidant potential [2], and biological antioxidants contain bioactive phytochemicals that may play a vital role in protecting the cell from oxidative stress [24]. Moreover, the effectiveness of potential antioxidants in tumor treatment may depend not only on the nature of the antioxidant, but also on the prevailing Po_2 in the tissues [1,9]. Thus, the choice of the antioxidants in this study was based on the following facts: (1) carotenoids are an effective chain-breaking antioxidant at low PO_2 [1,9], (2) vitamin E is an efficient antioxidant under high PO_2 [1] and (3) although vitamin C has been proposed as a chemotherapeutic agent since 1952, studies about its effects on cancer are still controversial [4]. Additionally, pequi oil has been used in popular medicine to treat a number of diseases [11], including tumor control [12]. However, this protective anticancer role needs to be further investigated, since there is only one study on the subject [25]. To this end, our study, which evaluates the effect of different antioxidant interventions on one type of tumor, can contribute to greater understanding of the potential effectiveness of these substances in cancer treatment.

Experimental studies support the role of ROS in the initiation and the promotion phase of carcinogenesis [1,2], in part, by showing that dietary antioxidants act as preventive agents of cancer [1]. Our results corroborate this evidence, since antioxidant administrations before tumor inoculation were effective in inhibiting tumor growth in the three experimental protocols. Among these groups, it was vitamin C that produced the greatest reduction. Because large solid tumors had extensive regions of internal necrosis, while in small tumors these regions were minor, the nonsignificant reduction of the necrosis area under the treatment with vitamin C was already expected. Moreover, vitamin C is considered the most important antioxidant in extracellular fluid [4], and significant reduction in DNA damage appeared only for antioxidant controls and vitamin C administered before tumor implantation, although pequi oil and vitamin E also showed nonsignificant reductions in this damage.

Because antioxidant enzyme activities fall in a wide variety of tumors [9,26–30], antioxidant administration may help the antioxidant system, inhibiting tumor growth by reducing ROS generation. Conversely, our results suggested that, while antioxidant administrations before tumor inoculation inhibit tumor growth, antioxidant administration after the tumor's appearance can accelerate tumor growth and favor the disease's progress, which is in accordance with the literature [31–33]. However, results also indicated that, if (and only if) preventively administered, pequi oil could be used as an adjuvant treatment in cancer therapy.

Since the 1930s, a number of researchers have observed that solid Ehrlich tumors develop in a high percentage of cases after routine intraperitoneal inoculations of ascitic fluid [34], but the reverse has not yet been reported. Because under favorable conditions, the Ehrlich tumor readily forms metastases [34], and as fluid Ehrlich tumors in other regions than the peritoneum have not yet been reported, mainly as a result of solid tumor metastasis, our results corroborate our previous suggestion. They also indicate that the efficiency and applicability of antioxidants in the medical clinic can depend not only on the nature of the antioxidant, the type and stage of cancer being treated [1,6,7] and the prevailing PO_2 in the tissues [1,9], but also on the type of antioxidant therapy chosen.

Although mice are able to synthesize ascorbic acid (vitamin C) in the liver and transport it to other parts of the body, and humans are naturally unable to synthesize this vitamin [4,35,36], it has been proposed that the concentration of ascorbic acid in blood regulates ascorbic acid synthesis. Exogenous ascorbic acid intake may influence the mechanism of biosynthesis, metabolism, elimination and storage of this vitamin in animals [35]. Additionally, it has been demonstrated that ascorbic acid plays a critical role in survival of the vasculature [36] and its depletion restricts angiogenesis and retards tumor growth in mice [37]. Thus, the controversial findings on the effectiveness of vitamin C treatment for cancer [4,5] were probably due to the different experimental procedures regarding the type of antioxidant therapy chosen, justifying once again the importance of the present study.

Biological antioxidants, such as those found in plant-based foods, contain bioactive phytochemicals and may play a vital role in protecting the cell from oxidative stress and cancer [24,38]. Plant-derived compounds have been an important source of several clinically useful anticancer agents [38], and several plants are known to exhibit antitumor properties [2,39–41]. Pequi oil is frequently used in regional cookery and in popular medicine to treat several illnesses [11], including tumor control [12]. Although this protective anticancer role has been reported in only one study from many years ago [25], other beneficial antioxidant and anti-

inflammatory effects of pequi fruit pulp oil have already been scientifically demonstrated by our group [42–45], corroborating its use in folk medicine and supporting the results obtained in this study.

It has been demonstrated that invading tumor cells advance through the submesothelial connective tissue spaces to extend the solid tumor [34], and similar histology was observed in this study with continuous administrations of vitamins E and C and with vitamin C administered after tumor inoculation. Such continuous administrations also led to increased anisocytosis, evaluated by RDW values, the most commonly reported index of the variation or degree of anisocytosis in cell volume within the red cell population [46]. Erythrocytes are very sensitive to oxidative stress because they are unable to repair damaged components, such as proteins, by resynthesis (nucleus absence), their membranes are vulnerable to peroxidative damage, and they have a poor repair mechanism [47,48]. Thus, results indicated that continuous administrations with vitamins E and C increased oxidative stress on RBC. Although values of RBC, HGB, hematocrit (HCT) and MCH differed significantly from the negative filtered water control in some antioxidant administration protocols, there were no significant differences between two specific groups in MCHC. As MCHC indicates, the mean concentration of HGB inside red cells (in respect to 1 dl of RBCs) [46], there was no significant alteration in the HGB population compared with the negative control.

The development of an ascites tumor is associated with production of inflammation [32] and, except for continuous administration with vitamin E, our results corroborated these findings, since neutrophils +monocytes significantly increased in the other groups, which presented ascites tumor metastasis. Results of the plateletgram corroborated results of the leukogram. Moreover, platelet volume reflects platelet reactivity [49], and in this context, the percentage of large platelets (platelet larger cell ratio or P-LCR) indicates greater reactivity for the pequi treatment before tumor inoculation and lower reactivity for the vitamin E control.

In summary, antioxidant administrations before tumor inoculation effectively inhibited tumor growth in the three experimental protocols, but administrations after the tumor's appearance accelerated its growth and favored metastases. Pequi oil given continuously inhibited tumor growth, while vitamins E and C accelerated it, favoring metastasis and increasing oxidative stress on RBC. Except under continuous administration of vitamin E, the development of ascites tumor metastases was related to increased inflammation. Thus, results suggest that the efficiency and applicability of antioxidants in the medical clinic can depend not only on the nature of the antioxidant, the type and stage of cancer being treated and the prevailing PO_2 in the tissues, but also on the type of antioxidant therapy chosen.

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