Article/Artigo

Vitamin C effects in mice experimentally infected with *Trypanosoma cruzi* QM2 strain

Efeitos da vitamina C em camundongos experimentalmente infectados com a cepa QM2 de *Trypanosoma cruzi*

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ABSTRACT

Introduction: To evaluate the efficacy of vitamin C in reducing the consequences generated by the production of free radicals in the acute and chronic phases of Chagas disease, two different doses of ascorbic acid were administered orally to 60 mice infected by *Trypanosoma cruzi* QM2 strain. **Methods:** The animals were divided into six groups: G1, G2, and G3 for the acute phase study, and G'1, G'2, and G'3 for the chronic stage. The groups G1 and G'1 received $8.6x10^{-4}$mg/g of vitamin C daily, whereas G2 and G'2 received $7.14x10^{-3}$mg/g daily. The other groups, G3 and G'3, were considered placebos and received 10µL of mineral water. **Results:** The study of the acute phase showed statistically significant differences between G1 and the other groups at various count days of the parasitemia evolution. The multiplying parasite was slower in G1 until the 11th day, but on the 22nd day it had greater parasitemia than in G2 and G3, and from the 36th day on, parasitemia stabilized at higher levels. However, when the histopathology of acute and chronic phases is considered, one does not note significant differences. **Conclusions:** The administration of two different doses of vitamin C was not able to protect mice and to contain the oxidative stress caused by free radicals formed by the metabolism of oxygen (reactive oxygen species) and nitrogen (reactive nitrogen species).

Keywords: Ascorbic acid. *T. cruzi.* Parasitemia. Free radicals.

RESUMO

Introdução: Para avaliar a eficácia da vitamina C em reduzir as consequências geradas pela produção de readicais livres na fase aguda e crônica da doença de Chagas, duas diferentes dosagens de ácido ascórbico foram administradas oralmente para 60 camundongos infectados pela cepa QM2 de *Trypanosoma cruzi*. **Métodos:** Estes animais foram divididos em seis grupos: G1, G2 e G3 para o estudo da fase aguda e G'1, G'2 e G'3 para o estudo da fase crônica. Diariamente, G1-G'1 recebeu $8.6 x 10^{-4}$ mg/g de vitamina C, G2- G'2 recebeu $7.14 x 10^{-3}$mg/g. Os outros grupos, G3-G'3, foram considerados placebos e receberam 10µL of de água mineral. **Resultados:** O estudo da fase aguda mostrou diferenças estatisticamente significativas entre G1 e os outros grupos em vários dias de contagens na evolução da parasitemia, e até o 11º dia a multiplicação parasitária foi menor em G1, mas no 22º dia ele tinha parasitemia maior que G2 e G3, e a partir do 36º, a parasitemia estabilizou em altos níveis. Quando considerado o histopatológico da fase aguda e crônica, não foi notado, entretanto, diferença significativa. **Conclusões:** Assim, foi encontrado que a administração de duas diferentes dosagens de vitamina C não foi capaz de proteger o camundongo e conter o estresse oxidativo causado pelos radicais livres formados pelo metabolismo do oxigênio (ROS) e nitrogênio (RNS).

Palavras-chaves: Acido ascórbico. *T. cruzi*. Parasitemia. Radicais livres.

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INTRODUCTION

According to the World Health Organization[1], approximately 13 millions individuals are infected by *Trypanosoma cruzi* in Central and South America, and every year 200,000 new cases are reported. According to the Ministry of Health, there are nearly three million cases in Brazil[2].

The only drug currently available for the treatment of these patients is benznidazole[3], the pharmacological action of which results in reactive oxygen species (ROS) formation as superoxide anion and hydrogen peroxide, promoting oxidative damage to *T. cruzi*[4-6]. Besides ROS, reactive nitrogen species (RNS), which are formed from the reaction of nitric oxide (NO) with superoxide radical[6,7], may also lead to oxidative stress, responsible for many pathological conditions such as chagasic cardiomyopathy[8].

Trypanosoma cruzi, however, seems to protect itself against the toxicity of free radicals by using ascorbic acid as an antioxidant, as suggested by Wilkinson et al.[9] and Monteiro et al.[10]. Wilkinson et al.[9] detected significant levels of ascorbate in epimastigotes of *T. cruzi*, both in free form and in oxidized form, dehydroascorbate.

To consider new perspectives for the treatment of Chagas disease, Maçao et al.[11] concluded that the administration of 500mg/day of vitamin C and 800UI/day of vitamin E for a six-month period was able to halt the progression of oxidative stress in the myocardium of patients at the chronic stage of Chagas disease, because vitamins C and E are important *sweepers* of free radicals[12].

Based on these studies, one would suppose that interference with the action of free radicals might influence the degree of blood proliferation of *T. cruzi* as well as the intensity of tissue injury in the target organs of Chagas disease.

As previous studies with vitamin C, free radicals, and *T. cruzi* were performed *in vitro,* with

biochemical parameters or genetic from parasite, we decided to check the levels of parasitemia and the anatomic-pathological features of trypanosomiasis using two different doses of ascorbic acid administered orally to mice infected with *T. cruzi* QM2 (*Quaraí-Macarrão 2*) strain, lineage IIc (TCIIc), both in the acute and the chronic phases of Chagas disease.

METHODS

Infection of mice

Sixty Swiss male mice from 20 days old were weighed to calculate the dosage of vitamin C, and they were intraperitoneally infected with $5.0x10^4$ trypomastigotes of *T. cruzi* QM2 strain[13], with blood from other mice previously infected. After infection, six groups of 10 mice were established at random, named G1, G2, and G3, and G'1, G'2, and G'3 for the study of the acute and the chronic phases, respectively. The animals were kept in individual cages to facilitate handling.

Calculation of the dosage of vitamin C and treatment

To calculate the dosage of vitamin C to be administered to infected mice, the daily dietary requirement of 60mg recommended for a man of 70kg body weight was considered[14,15] or approximately $8.6x10^{-4}$mg/g in weight. Vitamin C (ascorbic acid) was used, presented in 200mg/ml drops of mineral water for dilution.

G1 and G'1 received a daily dose of vitamin C D60 equal to $8.6x10^{-4}$mg, diluted in 10μL of mineral water. G2 and G'2 received a dose D500, corresponding to a daily dosage of 500mg/day (D500) equal to $7.14x10^{-3}$mg of vitamin C diluted in 10μL of mineral water. G3 and G'3 were considered placebos and received daily 10μL of mineral water.

Every day in the morning, all mice were treated orally and received in their mouth 10μL of vitamin C D60, D500, or mineral water with a Gilson automatic pipette. The mice in groups G1, G2, and G3 were treated for a period of 45 days to study the acute phase and those in group G'1, G'2, and G'3 for 180 days to study the chronic phase, starting from the infection date.

This study was approved by the Ethics Committee of the Faculty of Medicine of Marília (FAMEMA) under number 206/08. The care for the maintenance, treatment, and euthanasia of the mice followed the standards set by Colégio Brasileiro de Experimentação Animal/ Brazilian College of Animal Experimentation (COBEA) second Sogayar[16].

Study of parasitemia

Blood was collected from the tail following the Brener method[17], with correction of intermicroscopic field[18]. There was a first count of blood trypomastigotes on the 8th day post-infection, with subsequent assessments until the 43rd day, twice a week.

To analyze the parasitemia, descriptive data analysis and the following tests were used: Kolmogorov-Smirnov, Shapiro-Wilks, Fisher, and Student t test, with the exclusion criterion, the outliers boxplot prepared group to group for each day of counting. Significance level (α) was 5%.

Histopathologic study

For the histopathologic study a sample from the heart and skeletal muscle from the thigh of all mice were collected, on the 45th day of the study for the acute phase and on the 180th day of the study for the chronic phase. The tissues were embedded in paraffin, and 5μm sections were stained with hematoxylin-eosin and examined under a light microscope with a magnification of 400 times. For each fragment five sequential histological sections were performed, which were analyzed and graded for inflammation process and amastigotes nests, for a total of 10 high-magnification fields for each type of tissue.

We used a semi-quantitative scale from zero to three to grade the inflammatory process and the nests of amastigotes, where the first *zero* meant the absence of inflammation, *one* meant mild inflammation, *two* meant moderate inflammation, and *three* meant intense inflammation; the second *zero* meant no amastigote nests, *one* meant rare nests of amastigotes, *two* meant a moderate number of nests of amastigotes, and *three* meant frequent nests of amastigotes.

To analyze the histopathology, descriptive data analysis and the Kruskal-Wallis test (ANOVA non-parametric) with post-test were used. Significance level was 5%.

RESULTS

Figure 1 shows the parasitemia curve. All groups showed patent parasitemia on the 8th day post-infection, and on this day G2 had lower parasitemia that was statistically significant compared with G1 (**Table 1**).

The following counts of blood trypomastigotes showed great variability. Parasitic multiplication in G1 increased more slowly than in the other groups until the peak of parasitemia, which occurred on the 18th day for G2 and G3 and on the 22nd day for G1. Between the 8th day and the 18th day, parasitemia in G1 was lower than that in G2 and G3, with statistically significant results. Between the 32nd day and 43rd day, G1 parasitemia was statistically significantly greater, stabilizing at higher levels than in the other groups. Between G2 and placebo, no statistically significant difference was observed.

The histopathology of the acute phase showed a higher frequency of parasite nests and moderate to intense inflammation in skeletal muscle compared with cardiac muscle, but the differences were not statistically significant, as observed from non-parametric tests for comparison of medians. The isolated analysis of the heart showed

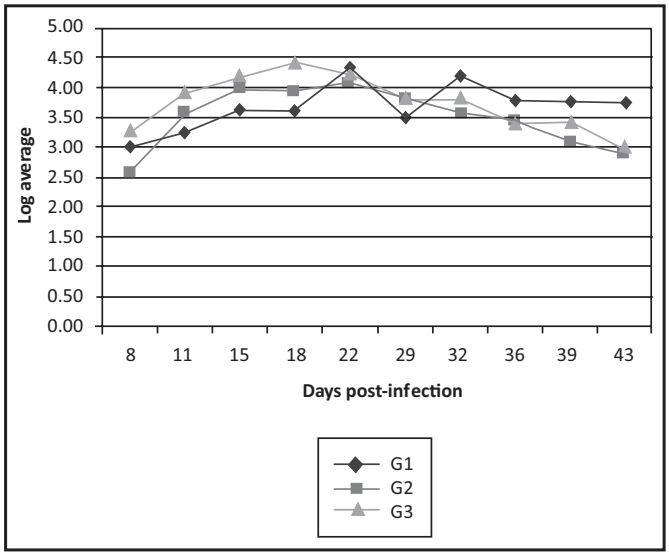

FIGURE 1 - Parasitemic curve of blood tripomastigotas/5μl number by log mean performed during the acute phase of *Trypanosoma cruzi* QM2 (*Quaraí-Macarrão* 2) strain infection in mice treated with two different doses of vitamin C and placebo.

TABLE 1 - Evolution of parasitemia during the acute phase of *Trypanosoma cruzi* infection in mice infected with *Trypanosoma cruzi* QM2 strain and treated with two different doses of vitamin C and placebo.

Days after infection	Group	Average parasitemia/5µL blood	Standard deviation	Compared groups	p-value (%)
Eight (8)	G1	995.45	1,173.83	G1 & G2	> 5.0
	G2	375.16	424.19	G1 & G3	4.8
	G3	1,810.95	1,898.29	G2 & G3	> 5.0
Eleven (11)	G1	1,782.06	1,733.76	G1 & G2	3.5
	G2	3,630.31	3,669.11	G1 & G3	> 5.0
	G3	8,101.64	6,996.39	G2 & G3	> 5.0
Fifteen (15)	G1	4,223.07	3,112.36	G1 & G2	> 5.0
	G2	9,457.59	7,961.51	G1 & G3	> 5.0
	G3	15,062.41	12,129.64	G2 & G3	> 5.0
Eighteen (18)	G1	4,094.27	2,819.40	G1 & G2	> 5.0
	G2	8,444.28	5,835.92	G1 & G3	> 5.0
	G3	26,759.63	26,326.31	G2 & G3	> 5.0
Twenty-two (22)	G1	21,187.36	18,328.97	G1 & G2	3.9
	G2	12,036.14	9,122.30	G1 & G3	4.3
	G3	16,882.90	10,999.47	G2 & G3	> 5.0
Twenty-nine (29)	G1	3,328.99	2,658.11	G1 & G2	> 5.0
	G2	6,425.68	3,835.54	G1 & G3	4.8
	G3	6,457.87	3,151.77	G2 & G3	> 5.0
Thirty-two (32)	G1	15,898.46	15,268.93	G1 & G2	3.5
	G2	3,761.57	3,741.75	G1 & G3	> 5.0
	G3	6,293.62	3,913.86	G2 & G3	> 5.0
Thirty-six (36)	G1	6,139.33	5,334.10	G1 & G2	> 5.0
	G2	2,799.03	2,241.90	G1 & G3	> 5.0
	G3	2,484.22	2,764.70	G2 & G3	> 5.0
Thirty-nine (39)	G1	6,211.39	6,170.41	G1 & G2	> 5.0
	G2	1,182.55	608.13	G1 & G3	> 5.0
	G3	2,719.79	2,411.29	G2 & G3	> 5.0
Forty-three (43)	G1	5,382.69	5,032.09	G1 & G2	3.9
	G2	810.99	329.44	G1 & G3	4.3
	G3	931.38	569.83	G2 & G3	> 5.0

QM2: *Quaraí-Macarrão* 2 strain.

that G1 had fewer parasites compared with G2 and placebo, but no statistically significant difference was observed (**Table 2**).

In the chronic phase there was a decrease in amastigote nests in both the heart and the skeletal muscle, but the inflammation was less intense in the heart of G2 compared with G1 and placebo.

TABLE 2 - Histopathologic analysis performed in the cardiac muscle and skeletal muscle during acute and chronic experimental chagasic infection by *Trypanosoma cruzi* QM2 strain in mice treated with two different doses of vitamin C and placebo.

		Average degree of lesion			
		cardiac muscle		skeletal muscle	
		amastigotes	inflammatory	amastigotes	inflammatory
Phase	Group	nests	process	nests	process
Acute	G1	0.40	1.40	0.80	2.30
	G2	0.70	1.40	0.80	2.20
	G3	0.90	1.70	0.70	2.40
Chronic	G1'	0.20	0.40	0.10	0.40
	G2'	0.00	0.10	0.10	0.50
	G3'	0.10	0.30	0.20	0.50

QM2: *Quaraí-Macarrão* 2 strain.

DISCUSSION

Vitamin C or ascorbate is a potent hydrosoluble antioxidant in biological systems *in vitro*[19] because it is an electron donor that can directly neutralize ROS[20]. Depending on the dosage, however, it can also act as a pro-oxidant[19,20], considering that ascorbate accelerates the glycation end-product formation by connecting the copper protein ascorbylated with free radicals generation by copper-protein complex[19].

Maçao[7] and Ribeiro[6] showed a decrease in oxidative stress in chronic patients with Chagas disease treated with vitamin C associated with vitamin E, indicating that the two vitamins act synergistically to prevent peroxidation. Carvalho et al.[21] showed that vitamin E deficiency caused exacerbation of sympathetic denervation in mice infected with *T. cruzi* in the acute phase of illness.

The survey results showed significant differences at times between the two dosages of vitamin C and placebo, as shown in **Table 1**, although none of the treated groups gained control of parasitemia and the inflammatory process. One should also consider the possible ability of the parasite to possess mechanisms to benefit from the action of ascorbic acid.

Several studies have demonstrated that *T. cruzi* can synthesize ascorbic acid from non-enzymatic and enzymatic systems and thereby protect themselves from ROS and RNS[10,22-24]. According to Wilkinsom et al.[23] parasites of the *Trypanosoma* genus may withdraw ascorbate from the culture medium and thus complement the deficiency of biosynthesis.

Jockers-Scherübl et al.[25], Wilkinsom et al.[23], Czechowicz et al.[26], and Ribeiro[6] reported the presence of one trypanothione reductase dependent on flavoenzyme nicotinamide adenine dinucleotide phosphate; this reductase catalyzes the reduction of trypanothione antioxidant, and this system is able to combat oxidative stress intracellular parasites. Studies by Krieger et al.[27] showed the possibility of the parasite of this enzyme to enhance its growth and virulence.

Another enzyme, 1-Cys-peroxiredoxins, described by Monteiro et al.[10], is also used by *T. cruzi* to synthesize ascorbate; this way, it can decompose peroxides to defend against the action of free radicals.

Although no similar experimental research like ours has been found, the administration of different doses of vitamin C did not alter the muscle tropism of QM2 strain, as amastigote nests in the heart and skeletal muscle were observed in three groups, but without statistically significant differences in histopathologic analysis. This histopathologic analysis was similar to that found by Martins et al.[13], who classified this strain in biodemes II after its isolation, according to criteria by Andrade[28].

Thus, despite the administration of two different doses of vitamin C, the histopathologic findings correlated with parasitemia and showed that the administration of vitamin C alone was not able to protect mice and was not likely to contain the oxidative stress caused by free radicals formed by the metabolism of oxygen (ROS) and nitrogen (RNS), which was observed by an inflammatory process presence similar in three groups, establishing the characteristic lesions of Chagas disease.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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