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# EFFECTS OF VITAMIN C SUPPLEMENTATION ON ACUTE PHASE CHAGAS DISEASE IN EXPERIMENTALLY INFECTED MICE WITH *Trypanosoma cruzi* QM1 STRAIN

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## SUMMARY

The tissue changes that occur in Chagas disease are related to the degree of oxidative stress and antioxidant capacity of affected tissue. Studies with vitamin C supplementation did not develop oxidative damage caused by Chagas disease in the host, but other studies cite the use of peroxiredoxins ascorbate - dependent on *T. cruzi* to offer protection against immune reaction. Based on these propositions, thirty "Swiss" mice were infected with *T. cruzi* QM1 strain and treated with two different vitamin C doses in order to study the parasitemia evolution, histopathological changes and lipid peroxidation biomarkers during the acute phase of Chagas disease. The results showed that the parasite clearance was greater in animals fed with vitamin C overdose. There were no significant differences regarding the biomarkers of lipid peroxidation and inflammatory process or the increase of myocardium in animals treated with the recommended dosage. The largest amount of parasite growth towards the end of the acute phase suggests the benefit of high doses of vitamin C for trypomastigotes. The supplementation doesn't influence the production of free radicals or the number of amastigote nests in the acute phase of Chagas disease.

**KEYWORDS:** Lipid peroxidation biomarkers; Chagas disease; Parasitemia; Inflammatory process; *T. cruzi;* Ascorbic acid.

## INTRODUCTION

In Brazil, approximately three million individuals are infected by *Trypanosoma cruzi*, with a predominance of chronic cases resulting from past infections. However, in recent years the occurrence of acute Chagas disease has been observed in states of Bahia, Ceara, Piaui, Sao Paulo and Santa Catarina, with a higher frequency of cases and outbreaks in some states of the Amazon Region[6].

In Chagas disease, it is observed that much of the damage to the host is caused by excess free radical production in tissues infected by *T. cruzi*. The reactive oxygen in the species metabolism (ROS) can damage any cell component, but the ones most affected are the cell membranes. In order to combat free radicals, aerobic biological systems use two mechanisms, one of which works with compounds such as reduced glutathione (GSH), superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and vitamin E, which has the ability to neutralize ROS before they cause tissue damage. The other mechanism is performed by ascorbic acid, glutathione reductase (GSH-Rd) and GSH-Px, which repair the damage caused by free radicals, reversing membrane lipid peroxidation[9].

The thiobarbituric acid reactive substances (TBARS) are compounds produced after membrane lipid peroxidation, and protein carbonyl is an oxidation product of cellular proteins. Both oxidative stress products are caused by Chagas disease. MAÇAO *et al*.[12] revealed reduced levels of TBARS and protein carbonyl in patients with Chagas cardiomyopathy who received supplemental vitamin C and E, showing a strong indication that these non-enzymatic antioxidants are effective in neutralizing the oxidative insult caused by Chagas disease.

Recent studies have shown that some parasites can decrease ascorbate-dependent antioxidative enzymes using the ascorbic acid present in infected tissues, and can therefore protect themselves from the oxidizing action of ROS and reactive nitrogen species (RNS) produced by host inflammatory cells[11,14]. In *Saccharomyces cerevisiae*, *Plasmodium falciparum* and *T. cruzi* peroxiredoxines (prx) type 1-Cys become active in peroxide detoxification when reduced by ascorbic acid. *T. cruzi* is lacking in catalase, superoxide dismutase and glutathione peroxidase selenocysteine-dependent and therefore depends on prx to decompose peroxides produced by host macrophages[11,14].

Based on these suggestions we propose the respective study in mice experimentally infected with *T. cruzi* QM1 strain in the acute phase of Chagas disease.

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## MATERIALS AND METHODS

**1. Infection of mice:** Thirty "Swiss" male mice, each 20 days old were intraperitoneally infected with 5.0 x $10^4$ trypomastigotes of *T. cruzi* QM1 strain, characterized by MARTINS *et al*.[13], using blood from other mice previously infected. Separated at random, three groups of 10 mice each were designated R, S, P. The Recommended group (R) received the recommended dose of vitamin C per gram of weight, the Overdose group (S) received an overdose of vitamin C per gram of weight and the Placebo group (P) received mineral water. The animals were kept in individual cages to facilitate handling and they were fed with Nuvilab CR-1® and water *ad libitum*.

**2. Calculation of the vitamin C dose and treatment:** Considering that the recommended daily dose of vitamin C for a man of 70,000 g is 60 mg[4,18], which results in approximately 8.6 x $10^{-4}$ mg of vitamin C per gram of weight, a D60 dose of vitamin C equal to 8.6 x $10^{-4}$ mg / g, dissolved in 10 µL of mineral water was orally given to group R daily. Since various brands available for sale provide a dose of 500 mg of vitamin C daily for a man of 70,000 g, which results in approximately 7.14 x $10^{-3}$ mg of vitamin C per gram of weight; a dose of vitamin C equal to 7.14 x $10^{-3}$ mg/g, dissolved in 10 µL of mineral water was administered orally to group S D500 on a daily basis. Group P received 10 µL of mineral water daily. Groups R, S and P received this treatment for 60 days. Each morning, all mice were treated and orally received 10 µL of vitamin C D60, D500 or mineral water using a Gilson automatic pipette.

The treatment form, care and euthanasia of mice was in accordance with Cobea rules[20]. This study was approved by the Ethics Committee of the Faculty of Medicine of Marília (FAMEMA), protocol nº 133/10.

**3. Study of parasitemia:** During 60 days the parasitemia of each mouse from groups R, S and P was determined twice a week following the BRENER[3] method, starting on the 7th day post- infection, and totaling 10 counts for each animal.

**4. Histopathological study:** For histopathological analysis a fragment of heart, colon and skeletal muscle tissue taken from the thigh of all mice of groups R, S and P was collected on the 60th day post- infection. The tissues were embedded in paraffin and 5 µm sections were stained with hematoxylin-eosin and examined under a light microscope with 400 times magnification. For each fragment five sequential histological sections were performed, which were analyzed and graded for inflammation process and amastigotes nests, a total of 10 high magnification fields were performed for each type of tissue.

A semiquantitative scale from zero to three was used to grade the inflammatory process, amastigote nests and necrosis. "Zero" was considered as – the absence of inflammation, necrosis and amastigote nests, "one" – mild inflammation, and rare amastigote nests and necrosis, "two" – moderate inflammation, moderate number of amastigote nests and necrosis, and "three" – intense inflammation, frequent amastigote nests and necrosis.

**5. Biochemical analysis:** Blood was collected by cardiac puncture into heparin, centrifuged at 1000 g and 200 µL plasma portion, and was immediately acidified with 800 µL of 5% trichloroacetic acid for subsequent ascorbic acid determination. Plasma and homogenate were stored at -80 ° C. One fragment of heart muscle was immediately frozen in liquid nitrogen and subsequently stored at -80 ° C.

Thiobarbituric acid reactive substances (TBARS), a lipid peroxidation biomarker, were placed in plasma and heart muscle using a method adapted from COSTA *et al*. (2006)[5]. A portion of homogenated plasma or muscle (100 µL) was mixed with 1 mL of a solution containing 15% (w/v) trichloroacetic acid, 0.38% (w/v) thiobarbituric acid and 0.25 mol/L of hydrochloric acid (HCl). The mixture was heated at 100 °C for 40 minutes, and the supernatant was read at 535 nm. Total concentration of TBARS was determined using the absorbance difference between samples and the standard solution of MDA.

Total peroxides, also a lipid peroxidation biomarker, were determined using the FOX method in cardiac muscle and plasma, as described by SÖDERGREN *et al*. (1998)[19]. A solution of 100 µmol/L xylenol orange, 4 mmol/L butylated hydroxytoluene, 25 mmol/L sulfuric acid and 250 µmol/L ammonium ferrous sulfate in a HPLC grade methanol:water (9:1 v/v) was added to 100 µL of plasma or muscle homogenate. The mixture was incubated for 30 min in darkness and at room temperature. Absorbance of the supernatant was measured at 560 nm. Total peroxides in muscle and plasma were determined comparing them with the standard $H_2O_2$ curve.

The ascorbic acid concentration in plasma and heart muscle was determined according to BESSEY (1960)[2]. 100 µL of solution containing 2,4-dinitrophenylhydrazine (2%), thiourea (5%) and copper sulphate (0.6%) in sulfuric acid (25%) was added to 300 µL of acidified plasma. After four hours incubation in a 37 ˚C water bath, 200 µL of sulfuric acid (65%) was added. The 20 min reaction was performed at room temperature and the reading was performed at 520 nm in a spectrophotometer (Spectramax M5, Molecular Device), and compared to a standard ascorbic acid curve.

**Statistical analysis:** The results of the biochemical analysis were analyzed by descriptive statistical methods and statistical inference using the Student's t test and Fisher. The significance level used was 0.05. In order to analyze the histopathologic, descriptive data analysis, the following tests were used: Kruskal-Wallis test (ANOVA non-parametric) with post-test. Significance level was 5%.

## RESULTS

**1. Study of parasitemia:** Figure 1 shows that all groups had parasitemia on the 7th day post-infection, and it appeared larger in group S than in groups R and P, with statistically significant results. However, from the 13th to the 40th day there was an increase observed in groups P and R, with more significant results on the 13th and 16th days than in group S. However, at the end of the acute phase, group S showed a tendency to stabilize the parasitemia in higher values than in groups R and P. It was observed that on the 50th day group P showed lower parasitemia than groups R and S with a significant difference. An isolated analysis of the groups R and P showed a statistically significant difference only on day 50th with higher parasitemia in group R.

**2. Histopathological analysis:** Table 1 shows that the three groups showed inflammation in all tissues examined, although at a lesser intensity in the heart muscle. Group R showed a stronger inflammatory process in

**Table 1**
Histopathological analysis in cardiac muscle, skeletal muscle and colon tissue during the acute phase of experimental infection by *T. cruzi* QM1 strain in mice treated with two different doses of vitamin C and placebo

| Groups | Cardiac Muscle | | Skeletal Muscle | | | Colon | |
|---|---|---|---|---|---|---|---|
| | Inflammation | Amastigote | Inflammation | Amastigote | Necrosis | Inflammation | Amastigote |
| P | 0.22 | 0.00 | 1.00 | 1.00 | 0.56 | 0.89 | 0.89 |
| R | 0.78 | 0.00 | 1.56 | 1.22 | 0.89 | 0.56 | 1.33 |
| S | 0.30 | 0.00 | 1.50 | 1.00 | 1.50 | 1.00 | 1.30 |

P = animals treated with 10 µL of mineral water; R = animals treated with D60 dose of vitamin C equal to 8.6 x $10^{-4}$ mg/g; S = animals treated with D500 dose of vitamin C equal to 7.14 x $10^{-3}$ mg/g.

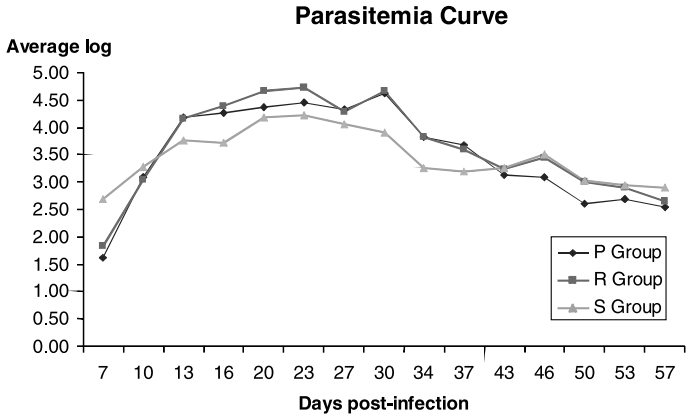


**Fig. 1 -** Parasitemia curve of blood trypomastigotes/5 µL number by log mean performed during the acute phase of *T. cruzi* QM1 strain infection in mice treated with two different doses of vitamin C and placebo.

heart muscle than groups P (p = 0.0167) and S (p = 0.0377). The presence of amastigote nests was observed in both skeletal muscle and colon tissue.

**3. Biochemical analysis:** The analysis in Tables 2 and 3 show that in the acute phase there was no statistical difference between groups in any analyzed parameters (p > 0.05 for all comparisons).

## DISCUSSION

According to WEYERS *et al.*[22] and RIBEIRO[17] adequate doses of vitamin C can act against lipid peroxidation caused by ROS formed naturally or by exogenous compounds in mice, because in electron donating the ascorbate chemically reduces lipid after the peroxidation process. However, the studies of PADAYATTY *et al.*[15] and FERREIRA & MATSUBARA[8] showed that depending on the vitamin C dose, it can act as pro-oxidant.

For LEVINE *et al.*[10], the plasma levels of vitamin C plasmatic are maintained when dose fractionation occur at regular intervals of less than 1.5 h. For BENKE[1] the plasma concentration of ascorbic acid depends on the dose and administration method, it reaches a saturation plateau within 3 hours of 1g vitamin C having been administered orally and decays linearly within 24 hours. The research of PADAYATTY *et al.*[16] performed on human plasma did not show significant differences when different doses of vitamin C were administered orally, whereas a significant increase in plasma levels was observed when there was an intravenous application of same dosages.

The researches above could explain the results obtained in this present study where the concentration of vitamin C in plasma and tissue was similar in all groups, without significant difference between groups R and S in comparison with group P, probably because mice supplementation had been performed orally in the morning, in a single daily dose and plasma and tissues collection for this analysis had been done at the end of the 60th day.

**Table 2**
Plasma levels of TBARS, total peroxide and ascorbic acid of mice treated with two different doses of vitamin C and placebo during the acute phase of experimental infection by *T. cruzi* QM1 strain. Variables were expressed as mean ± standard deviation

| Variables | P | R | S |
|---|---|---|---|
| TBARS (µmol/mL) | 50.1 ± 7.0 | 51.9 ± 11.5 | 54.7 ± 6.4 |
| Total peroxide (Mmol/$H_2O_2$ equivalent) | 104.5 ± 35.9 | 139.9 ± 50.0 | 99.9 ± 28.6 |
| Ascorbic acid (mg/dL) | 0.26 ± 0.05 | 0.29 ± 0.14 | 0.24 ± 0.05 |

P = animals treated with 10 µL of mineral water; R = animals treated with D60 dose of vitamin C equal to 8.6 x $10^{-4}$ mg/g; S = animals treated with D500 dose of vitamin C equal to 7.14 x $10^{-3}$ mg/g.

**Table 3**
TBARS, total peroxide and ascorbic acid in mice miocardium treated with two different doses of vitamin C and placebo during the acute phase of experimental infection by *T. cruzi* QM1 strain. Variables were expressed as mean ± standard deviation

| Variables | P | R | S |
|---|---|---|---|
| TBARS (µmol/g prot) | 301.1 ± 74.7 | 298.0 ± 83.9 | 359.1 ± 72.8 |
| Total peroxide (mmol $H_2O_2$ equivalent/ g prot) | 528.8 ± 112.0 | 637.8 ± 130.6 | 538.2 ± 70.8 |
| Ascorbic acid (µg/g prot) | 5.10 ± 0.51 | 5.80 ± 1.90 | 6.36 ± 4.02 |

P = animals treated with 10 µL of mineral water; R = animals treated with D60 dose of vitamin C equal to 8.6 x $10^{-4}$ mg / g; S = animals treated with D500 dose of vitamin C equal to 7.14 x $10^{-3}$ mg / g.

Considering the researches cited earlier and how the animals studied were treated, we may conclude that vitamin C did not interfere in ROS action and production, as would appear to be the case in the histopathological analysis, which showed a similar amount of inflammation in skeletal muscle and colon tissue in groups R and S as in group P. The reduced inflammation and absence of amastigote nests in the heart may be related to a strain characteristic as well as the fact observed by WEN *et al*.[21] and DI MEO *et al*.[7] who found an improved antioxidant defense system in the hearts of normal mice in comparison with their skeletal muscle and colon tissue. The lower antioxidant efficiency of skeletal muscle may be one explanation for the occurrence of larger damage to this tissue in the acute phase of the illness in this work.

Similarly, the multiplication of the parasite was not affected by different doses of vitamin C, which can be observed by the finding of similar numbers of amastigote nests in the skeletal muscle and colon tissue among the three groups. According to MONTEIRO *et al*.[14] the parasite has mechanisms which allow it to benefit from the action of ascorbic acid, it can also use the plasmatic ascorbate present in mammalians to reduce parasite prx and thereby protect itself from peroxides produced by the host, despite that LOGAN *et al.*[11] showed that although *T. cruzi* is not able to capture extracellular vitamin C to maintain their enzymes prx active, it is able to synthesize vitamin C from galactono-γ-lactone and D-arabino-γ-lactone.

At this stage of the disease it was not observed total peroxides and not decreasing TBARS levels in groups R and S compared to P group, although studies with vitamin E supplementation (800 IU / day) and vitamin C (500 UI/day) had shown TBARS levels reduction in patients with the chronic form of Chagas' disease[12]. The non-animal supplementation with vitamin E in this study may have been the reason why TBARS levels did not reduce in groups R and S, because vitamins C and E act synergistically, blocking lipid peroxidation propagation in cell membranes[12].

We observed that group S parasitemia was lower than in groups P and R from the 10$^{th}$ to the 43$^{rd}$ day, suggesting that the vitamin C overdose orally administered to this group possibly allowed the immune system to temporarily take contol of parasitic multiplication in the blood, but not in other tissues, indicating that a single daily administration of vitamin C was not enough to maintain the concentration needed to avoid oxidative stress. Moreover, the fact that group S had a higher parasite growth at the end of the acute phase suggests that trypomastigotes can benefit from this higher dosage, as proposed by MONTEIRO *et al*.[14] and LOGAN *et al*.[11].

## RESUMO

### Efeitos da suplementação de vitamina C sobre a fase aguda da doença de Chagas em camundongos experimentalmente infectados pela cepa QM1 de *Trypanosoma cruzi*

As alterações teciduais que ocorrem na doença de Chagas estão relacionadas ao grau de estresse oxidativo e à capacidade antioxidante do tecido afetado. Estudos realizados com suplementação de vitamina C revelaram redução no dano oxidativo causado no hospedeiro pela doença de Chagas, porém outros estudos citam o uso de peroxiredoxinas dependentes de ascorbato pelo *T. cruzi* para se proteger da ação imune. Com base nessas proposições, trinta camundongos "Swiss" foram infectados com a cepa QM1 de *T. cruzi* e tratados com duas diferentes doses de vitamina C para estudar a evolução da parasitemia, alterações histopatológicas e dosagem de biomarcadores de peroxidação lipídica durante a fase aguda da doença de Chagas. Os resultados mostraram que a parasitemia foi maior nos animais que receberam uma superdosagem de vitamina C. Não houve diferenças significativas quanto aos biomarcadores de peroxidação lipídica e houve maior processo inflamatório no miocárdio dos animais tratados com dosagem recomendada. O maior crescimento parasitário ao fim da fase aguda sugere benefício de altas doses de vitamina C aos tripomastigotas. A suplementação não exerceu influência sobre a produção de radicais livres e o número de ninhos de amastigotas na fase aguda da doença de Chagas.

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## CONFLICT OF INTEREST

All authors declare no conflicts of interest in developing this study.

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