

Action of the medicine Canova[®] on peritoneal resident macrophages infected with *Trypanosoma cruzi*

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ABSTRACT. Approximately 20 million of people are chronically infected with *Trypanosoma cruzi* in Latin America. The present work investigated the action of the homeopathic medicine Canova[®] on *in vitro* experimental infections with *T. cruzi* Y strain, using Swiss mice resident peritoneal macrophages. Our results demonstrated that Canova[®] induced a decrease in the production of H₂O₂ and TNF- α at 20 and 40% concentrations when compared to the control RPMI. However, when compared with this medicine excipient, a significant decrease in these mediators was observed with Canova[®] at 40% concentration only. The production of NO and phagocytic activity were not affected. TNF- α inhibits *T. cruzi* replication in peritoneal macrophages *in vitro*, becoming an important agent of infection control by this parasite. Within this context, Canova[®], unlike what has been reported with other infections, would function as a stimulator of the infection, since it inhibited the production of TNF- α by peritoneal resident macrophages *in vitro*. Further studies should be carried out with elicited macrophages, in order to confirm the inhibitory activity of Canova[®] on the production of TNF- α and other mediators in macrophages infected by *T. cruzi*.

Key words: homeopathy, *Trypanosoma cruzi*, Canova[®].

RESUMO. Ação do medicamento Canova[®] em macrófagos peritoniais residentes infectados por *Trypanosoma cruzi*. Aproximadamente 20 milhões de pessoas são cronicamente infectadas pelo *Trypanosoma cruzi* na América Latina. O presente trabalho investigou a ação do medicamento homeopático Canova[®] em infecções experimentais “*in vitro*” com *Trypanosoma cruzi*, cepa Y, usando macrófagos residentes peritoniais de camundongos Swiss. Os resultados indicaram que Canova[®] induz a diminuição significativa da produção de H₂O₂ e TNF- α em concentrações de 20 e 40%, quando comparado com o controle RPMI. Quando comparado com o excipiente do medicamento, observou-se diminuição na concentração destes mediadores apenas na concentração de 40%. A produção de NO e a atividade fagocítica não foram afetadas. TNF- α inibe a replicação do protozoário em macrófagos peritoniais “*in vitro*”, mostrando-se importante agente para o controle da infecção pelo parasita. Portanto, o medicamento Canova[®] poderia estimular o processo de infecção, pois promoveu inibição da produção de TNF- α por macrófagos peritoniais residentes “*in vitro*”. Estudos adicionais devem ser realizados com macrófagos elicitados, a fim de confirmar a atividade inibitória da Canova[®] sobre a produção de TNF- α e outros mediadores em macrófagos infectados por *T. cruzi*.

Palavras-chave: homeopatia, *Trypanosoma cruzi*, Canova[®].

Introduction

Chagas disease is caused by the intracellular protozoan *Trypanosoma cruzi* (Chagas, 1911), a parasite of many mammal species. *T. cruzi* invades a variety of types of host cells, circulates in the blood stream in the form of a trypomastigote and proliferates inside the cytoplasm of those cells, as amastigote. The rupture of the amastigotes causes

cellular destruction and an intense inflammatory process, especially in the heart (Cummings and Tarleton, 2004).

It is estimated that 18 to 20 million of people are chronically infected with *T. cruzi* in Latin America (Ropert *et al.*, 2002). This protozoan infects mainly the macrophages, which play a role in infection control (Venkata and Gangadharam, 1992). When a

macrophage is stimulated, it modifies its adhesion property, its endocytic rates, and its fusion with endocytic vacuoles (Cunningham and Kuhn, 1980), improving its phagocytic rate and microbicidal activity (Anosa et al., 1997). Therefore, at first, the infection is immunosuppressed by the macrophages through the production of several cytokines, causing the death of the amastigotes forms both *in vitro* (Plata et al., 1984; Wirth and Kierszenbaum, 1988), and *in vivo* (Reed et al., 1987).

One of the cytokines involved in the microbicide activity by the macrophages is the tumor necrosis factor alpha (TNF- α) (Silva et al., 1995). Low concentrations of this cytokine induce the expression of adhesion receptors, favoring leukocyte adherence; on the other hand, in high concentrations it causes the development of pathological conditions, such as cachexia, septic shock and autoimmune disorders (Piemont and Buchi, 2002). Nitrogen intermediates produced by cytokine-activated macrophages, such as nitric oxide (NO), may also play a role in *T. cruzi* infection control (Deng et al., 1993).

Besides the organism's immune defenses, there are medicines that are used to control *T. cruzi* infection, some of synthetic origin, others natural. Canova[®] consists of a homeopathic, non-mutagenic, non-toxic drug, highly diluted and dynamic, which is indicated in pathologies where the individual's immune system is compromised, especially AIDS and cancer (Seligmann et al., 2003). Its active components are *Aconitum napellus*, *bryonia Dawn*, *Lachesis trigonocephalus* and *Thuya occidentalis* (Gabriel, 2004). As observed by Piemont and Buchi (2002), the macrophages treated with this drug become larger, with more cellular projections and a substantial increase of cytoplasmic volume; and according to Pereira et al. (2005), they cause low infection rates in macrophages infected *in vivo* by *Leishmania (L) amazonensis*.

In this work, we evaluated the effect of the Canova[®] drug on macrophages infected by *T. cruzi* *in vitro*, observing phagocytic activity of *T. cruzi* by macrophages treated with Canova[®], as well as the production of macrophage mediators.

Material and methods

Experimental outline. To carry out this study, we co-cultivated suspensions of Swiss mice resident peritoneal macrophages with and without *Trypanosoma cruzi* Y strain, in the presence of Canova[®] at 20 and 40% concentrations. As control, the Canova[®] medicine was replaced by RPMI 1640

media or by Canova's excipient (99% distilled water and 1% cereal alcohol) in the same concentrations. The culture supernatants were assessed for the production of NO and TNF- α . Other parameters assessed in the cultivated macrophages were H₂O₂ production and the *T. cruzi* phagocytic index. The experiments were carried out in triplicate or quadruplicate.

Mice and parasites. The macrophages used in the experiments were collected from 30 days Swiss mouse peritoneals, raised at the Central Bioterium of the State University of Maringá, Paraná State, Brazil. The *T. cruzi* Y strain used in the experiments was obtained from the Basic Parasitology Laboratory of the same university.

Canova[®]. Canova[®] preparation is described in the Brazilian Homoeopathic Pharmacopoeia (CPRFB, 1997; Lacerda, 1998). Starting from the original mother tincture (in the case of a plant this is an alcoholic extract) several dinamizations are performed. The final product, Canova[®], contains 19 dH *Thuya occidentalis* (Cupresaceae) made from the bark; 18 dH *Bryonia alba* (Cucurbitaceae) made from fresh roots; 11 dH *Aconitum napellus* (Ranunculaceae) made from fresh preparations of the whole plant, including the roots, at the beginning of flowering; 19 dH *Arsenicum album* (arsenic trioxide); and 18 dH *Lachesis muta* (Viperidae) venom (Pereira et al., 2005). dH units were used to describe the doses employed; one dH unit is a one-tenth dilution; thus, 10 dH represents 1×10^{-10} . The Canova[®] final product is an alcoholic extract diluted in 99% distilled water and 1% solution of cereal alcohol.

Obtaining *T. cruzi* blood trypomastigotes. High-infectivity *T. cruzi* Y strain blood trypomastigotes to be used in the *in vitro* studies were obtained after the establishment of a *T. cruzi* parasitemia curve in Swiss mice. The parasitemia curve was determined by the inoculation of 10,000 blood trypomastigotes in Swiss mice. Evaluation took place from the 3rd day of infection by the exam of 5 μ L of fresh blood between the sheet and glass coverslip, using the Brenner technique (Brenner, 1962). The parasitemia was assessed until the 30th day of infection. A curve was drawn using the mean counting of the inoculated animals to establish the parasitemia peak, which occurred on the 7th day after infection.

Cell cultures. Swiss mice resident peritoneal macrophages were cultivated in supplemented RPMI medium (RPMI 1640 GIBCO, Grand Island, NY, USA + 10% fetal bovine serum GIBCO, Grand Island, NY, USA) at 1×10^6 cell mL⁻¹ in 24 well plates. These plates were incubated for 2 hours

and then washed twice to remove the non-binding cells. The macrophage cultures were further incubated for 24 hours at 37°C and 5% CO₂ atmosphere for cell adaptation. Afterwards, Canova[®] at 20 and 40% concentrations, and blood trypomastigotes *T. cruzi* Y strain at a 2:1 parasite/macrophage ratio were added, and incubated for 2 hours. The supernatants were then removed and stored for subsequent NO and TNF- α evaluation. Control cultures were established in the same conditions of the drug excipient (99% of distilled water + 1% of cereal alcohol, at 20 and 40% concentrations), and with RPMI supplemented medium.

Determining the phagocytic index (PI). In order to determinate the phagocytic index, we cultivated Swiss mice resident peritoneal macrophages in the same conditions as previously described in 24-well plates containing a 13 mm-slide (Glastécnica). The supernatants were then removed and the slides were washed in PBS, stained by HEMA 3 - Stain (Biochemical Science, Inc., USA) and mounted with Enthellan (Merck) in glass sheets (24 x 76 mm). After staining, the slides were examined under optical microscope to determinate the percentage of infected macrophages and the number of parasites per macrophages (300 macrophages per slide). Based on these data, we calculated the phagocytic index through the formula $PI = \% \text{ of infected macrophages} \times \text{average number of phagocytosed parasites}$, according to Buchi and Souza (1992; 1993).

Determining nitric oxide (NO). The concentration of NO released by the treated and non-treated macrophages was measured in the supernatant of the cultures, through a colorimetric test ($\lambda = 550 \text{ nM}$) based on the Griess Reaction (Ding *et al.*, 1988). In brief, 50 μL of the culture supernatants was added to the 96-well culture plates, to which 50 μL of the Griess reagent was added (1% sulfanilamide, 0.1% 2-hydrochlorite naphthyl l- ethylenediamine, 2.5% orthophosphoric acid). The mixture was incubated for 10 to 15 minutes at room temperature. The reactions were made fourfold and the results were expressed in $\mu\text{M mL}^{-1}$, based on the Sodium Nitrite (NaNO₂) pattern curve.

Determining hydrogen peroxide (H₂O₂). To assess the production of H₂O₂, resident peritoneal macrophages (10⁵ cells) were cultivated as previously described, but in 96-well plates. The supernatant was then removed and 100 μL of phenol red buffer containing peroxidase was added to the macrophage cultures, and further incubated at 37°C for one hour in a humidified chamber. The reaction was interrupted with 10 μL of NaOH 1 M. The phenol

red oxidation was quantified by absorbance reading in a 620 nM wavelength. The production of hydrogen peroxide was determined with reference to a standard curve. The results were presented in nmoles of H₂O₂ 10⁻⁵ cells.

Determining tumor necrosis factor alpha (TNF- α). The TNF- α cytokine concentrations were assessed in the culture supernatants through the immunoenzymatic test ELISA (Enzyme Linked Immunosorbent Assay-ELISA) with pairs of antibodies from R&D System, Inc. (Minneapolis-USA). The technique was developed according to the manufacturer's protocols, with small changes. In short, 96-well plain bottom ELISA microwells (Nunc-MaxiSorp) were pre-coated with monoclonal anti-cytokine antibody and incubated for 18 hours at 4°C. The unspecified sites were blocked with skimmed powdered milk (Molico) dissolved in PBS-Tween (0.5 mL of tween-20 per liter) for 2 hours at 37°C. The culture supernatants were then added and incubated for 4 hours at 37°C. Polyclonal anti-cytokines biotinylated antibodies, used as capture antibodies, were added and incubated further for 2 hours. Next, a solution containing streptavidine HRP (Horse Radish Peroxidase) at 1:10.000 in PBS-Tween was added, and incubated for 2 hours at 37°C. After each incubation period, the plates were washed with PBS-Tween 3 to 5 times. The final reaction was developed with a TMB Solution (Tetra Methyl Benzidine) containing H₂O₂. The reading was made in 492 nM. The cytokine concentrations were determined with reference to a standard curve obtained with the recombinant murine cytokine (R&D System). The results were expressed in pg mL⁻¹.

Statistical analysis. The comparison between the groups was carried out by statistical tests (Student's t-Test and Main Whitney). We used the JandelSigmaStat software, version 2.0 (Statistical Software Jandel Corporation), to perform the analysis. *P* values equal to or smaller than 0.05 were considered significant.

Results

Effect of Canova[®] on the production of H₂O₂

As it can be seen in Table 1, Canova[®] at 20 and 40% concentrations promoted a significant decrease in the production of H₂O₂ by the Swiss mice resident peritoneal macrophages in the presence of *T. cruzi* Y strain, when compared to the control RPMI + *T. cruzi* (0.9 \pm 0.18 vs. 1.2 \pm 0.08, *P* =

0.024; 0.9 ± 0.13 vs. 1.2 ± 0.08 , $P = 0.003$, respectively at 20 and 40%). However, when we compared the effect of Canova[®] with its excipient in the same concentrations, we only observed a decrease in the production of H₂O₂ at the 40% concentration (0.9 ± 0.13 vs. 1.4 ± 0.09 , $P = 0.001$). A similar pattern was observed with macrophages not stimulated by *T. cruzi* (Table 2).

The experiments were not designed to compare the response of macrophages not infected with macrophages infected with *T. cruzi*. Nevertheless, when we compare data from Table 1 with Table 2, the *T. cruzi* infection by itself does not seem to affect the H₂O₂ level.

Canova[®] effect on the production of TNF- α

A significant decrease in the production of TNF- α was observed with Canova[®] at 20 and 40% concentrations in the presence of *T. cruzi* when compared to the control RPMI + *T. cruzi*. (117.7 ± 13.30 vs. 158.9 ± 8.23 , $P = 0.026$; 98.4 ± 0.32 vs. 158.9 ± 8.23 ; $P = 0.013$, respectively at 20 and 40%). Again, when we compare the effect of Canova[®] with its excipient at the same concentrations, the decrease in the production of TNF- α was kept only at the 40% concentration (98.4 ± 0.32 vs. 117.0 ± 0.85 , $P = 0.005$) (Table 1).

In the absence of *T. cruzi*, Canova[®] did not affect the production of TNF- α by resident peritoneal macrophages (Table 2). On the other hand, *T. cruzi* by itself seems to stimulate the production of TNF- α by resident peritoneal macrophages (158.9 ± 8.23 vs. 108.2 ± 18.23 , $P < 0.05$; Table 1 vs. Table 2), which is restored to basic levels when the infected macrophages are treated with Canova[®] at 20 and 40% (117.7 ± 13.3 and 98.4 ± 0.32) (Table 1).

Canova[®] effect on the production of NO

As for the production of nitric oxide, we did not observe any effect of Canova[®], neither in the presence (Table 1) nor in the absence of *T. cruzi* (Table 2). *T. cruzi* by itself in this culture conditions also did not seem to induce NO production by resident peritoneal macrophages (Table 1 vs. Table 2).

Canova[®] effect on the *T. cruzi* phagocytic index

Canova[®] in 20 and 40% concentrations did not affect the *T. cruzi* *in vitro* phagocytic activity by Swiss mice resident peritoneal macrophages, either when comparing with the control RPMI medium (51.2 ± 12.49 vs. 58.9 ± 5.10 , $P = 0.667$; 46.7 ± 14.17 vs. 58.9 ± 5.10 , $P = 1.00$, respectively), or with the Canova[®] excipient at the same concentrations (51.2 ± 12.49 vs. 48.3 ± 18.39 ,

$P = 0.667$ and 46.7 ± 14.17 vs. 42.4 ± 0.24 , $P = 1$, respectively) (Table 1).

Table 1. Effect of Canova[®] on the phagocytic index and on the production of H₂O₂, TNF- α , and NO by Swiss mice resident peritoneal macrophages infected with *Trypanosoma cruzi* Y strain trypomastigotes.

	RPMI	Canova [®] excipient (20%)	Canova [®] excipient (40%)	Canova [®] (20%)	Canova [®] (40%)
PI	58.9 ± 5.10	48.3 ± 18.39	42.4 ± 0.24	51.2 ± 12.49	46.7 ± 14.17
H ₂ O ₂ nM mL ⁻¹	1.2 ± 0.08	0.9 ± 0.18	1.4 ± 0.09	0.9 ± 0.18*	0.9 ± 0.13**/**
TNF- α pg mL ⁻¹	158.9 ± 8.23	105.1 ± 11.0	117.0 ± 0.85	117.7 ± 13.3*	98.4 ± 0.32**/**
NO μ M mL ⁻¹	24.4 ± 1.11	24.6 ± 1.05	28.3 ± 1.26	24.6 ± 1.05	25.6 ± 1.36

PI = phagocytic index (% of macrophages infected X average number of phagocytosed parasites); H₂O₂ = peroxid hydrogen; TNF- α = Tumor Necrosis Factor alpha; NO = nitric oxide; Canova[®] excipient = 99% distilled H₂O + 1% cereal alcohol; * $P = 0.003$ when compared to RPMI; ** $P < 0.001$ when compared to Canova[®] excipient at 40%; *** $P = 0.024$ when compared to RPMI; * $P = 0.026$ when compared to RPMI; ** $P = 0.013$ when compared to RPMI; *** $P = 0.005$ when compared to Canova[®] excipient at 40%.

Table 2. Effect of Canova[®] on the *in vitro* production of H₂O₂, TNF- α , and NO by Swiss mice resident peritoneal macrophages.

	RPMI	Canova [®] excipient (20%)	Canova [®] excipient (40%)	Canova [®] (20%)	Canova [®] (40%)
H ₂ O ₂ nM mL ⁻¹	1.5 ± 0.06	0.9 ± 0.21	1.0 ± 0.13	1.1 ± 0.09**/**	0.8 ± 0.03**
TNF- α pg mL ⁻¹	108.2 ± 18.23	98.0 ± 10.49	114.3 ± 19.61	105.4 ± 11.35	112.6 ± 10.12
NO μ M mL ⁻¹	27.6 ± 0.37	29.9 ± 4.40	27.8 ± 3.39	33.4 ± 4.02	28.4 ± 5.53

H₂O₂ = peroxid hydrogen; TNF- α = Tumor Necrosis Factor alpha; NO = nitric oxide; Canova[®] excipient = 99% distilled H₂O₂ + 1% alcohol of cereals; * $P = 0.037$ when compared to Canova[®] excipient at 20%; ** $P < 0.001$ when compared to RPMI.

Discussion

Macrophages work as defense cells against infections by *T. cruzi* and other intracellular microorganisms. The participation of those cells is fundamental to control this type of infection, and it can occur directly or through the production of cytokines and other mediators.

We observed in this work that Canova[®] at 40% promoted a decrease in the production of TNF- α and H₂O₂ by macrophages infected with the *T. cruzi* Y strain. Moreover, although the data are preliminary, we observed that mouse peritoneal macrophages infected with *T. cruzi* Y strain enhance their TNF- α production, which is restored to basic levels by Canova[®] treatment. Piemont and Buchi (2002) also showed in a different system the Canova[®]'s TNF- α inhibitory production effect over peritoneal macrophages. TNF- α alone inhibits both the *in vitro* and *in vivo* replication of *T. cruzi* in peritoneal or spleen macrophages, becoming an important agent of the innate and specific immune responses to control the infection by this parasite (Titto et al., 1986; Silva et al., 1995; Abrahamsohn

and Coffman, 1996). In this context, Canova®, differently from what has been reported for other infections (Pereira *et al.*, 2005), would act as infection stimulator, since it inhibited the production of TNF- α *in vitro* by peritoneal macrophages infected by *T. cruzi*. These results are in agreement with the effect of Canova® on the enhancement of the infection by *T. cruzi in vivo* (Pupulin *et al.*, 2004) and with the serum TNF- α lower level in the late stage of infection of animals treated with Canova® and benznidazol (Pupulin *et al.*, 2004).

On the other hand, H₂O₂ does not seem to have an important role in the infection control by this parasite. Melo *et al.* (2003) showed that the *in vivo* infection by *T. cruzi* does not induce the release of H₂O₂ by peritoneal macrophages on the initial phase of infection. The decrease in the production of H₂O₂ observed here, therefore, may just be a reflex of the *T. cruzi* + Canova® - induced inhibition of macrophage activation.

According to Oliveira *et al.* (2002), the homeopathic drug Canova® may stimulate the natural and acquired immune response through macrophage activation, as macrophages infected by different microorganisms have an increment in their phagocytic capacity when submitted to treatment with this drug. Similarly, Buchi and Souza (1992) demonstrated that macrophages of mice treated with Canova® *in vivo* and *in vitro* in several concentrations show an increase on the phagocytic activity of some intracellular parasites, such as *Leishmania amazonensis*, and yeasts such as *S. cerevisiae*. On the other hand, Abrahamssohn and Coffman (1996) stated that the intracellular killing of *T. cruzi* by macrophages is critically dependent on macrophage cytokine activation.

However, the present study did not demonstrate any effect of Canova® on the *in vitro* phagocytic activity of Swiss mice resident peritoneal macrophages stimulated with *T. cruzi* Y strain blood trypomastigotes. The same results were found by Lopes *et al.* (2006) with peritoneal macrophages stimulated *in vitro* for more than 24 hours with Canova®. Interestingly, the macrophage phagocytic activity over *T. cruzi* Y strain epimastigotes was enhanced by this drug by about 2 times. Altogether, the data indicate that the differences seen in the literature about the effect of Canova® may be linked to the different etiological agents used to evaluate the phagocytic activity.

As *T. cruzi* blood trypomastigotes infect macrophages actively, its internalization by the macrophages may be just a consequence of the

action of the *T. cruzi* itself, which is related to its virulence. In that case, the Canova® effect upon the phagocytic cell would not affect the level of parasite internalization. Moreover, in our work we carefully compared the effect of Canova® with its excipient at the 20 and 40% concentrations, since the drug excipient itself may affect the response of resident peritoneal macrophages on *T. cruzi* and other pathogens.

Nitric oxide has been described as an important mediator of the immune response to intracellular pathogens, presenting direct toxic effects as well as inhibiting parasitic multiplication. Some pathogens, including *T. cruzi*, have developed mechanisms of NO production inhibition, in order to promote their survival in the phagocytic cells (Piemont and Buchi, 2002). Our data showed that *T. cruzi* Y strain trypomastigotes alone, or in the presence of Canova®, and in the culture conditions here established, were not capable of changing the NO production by the Swiss mice resident peritoneal macrophages. Bergeron and Oliver (2006) also showed that both J774.2 cell line and BALB/c bone marrow-derived macrophages do not produce NO when stimulated with *T. cruzi* Y strain trypomastigotes alone from 3 to 24 hours and at various *T. cruzi*/macrophage proportions. Tatakijara *et al.* (2008) showed that resident C57BL/6 mice peritoneal macrophages, but not BALB/c mice macrophages, infected *in vivo* with *T. cruzi* trypomastigotes for 12 days presented an enhancement of NO production after 24 hours in culture. On the other hand, Pereira *et al.* (2005) showed that elicited BALB/c peritoneal macrophages treated with Canova® *in vitro* for 24 hours enhanced their NO production. Macrophages vary in respect to NO release, and response heterogeneity may be related to both the mouse strain studied and the heterogeneity of the triggering stimulus, combined with availability of different innate and adaptive immune response cytokines. Further studies with other culture protocols should be carried out to confirm these data. Again, the importance of using appropriate controls is emphasized in these experiments, so that the conclusions are trustworthy.

Final considerations

Overall, the data suggest that in the culture conditions here established Canova® induce inhibition of TNF- α and H₂O₂ release by macrophages infected with *T. cruzi* trypomastigotes. On the other hand, no release and phagocytosis were not affected. Further studies should be carried

out with elicited macrophages in order to confirm the TNF- α production inhibition. In this context, it is important to point out that homeopathic drugs, similarly to allopathic drugs, can produce undesirable effects; therefore, they should be administered carefully and after extensive studies.

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