Effect of Citrinin and in Association with Aflatoxin B_1 on the Infectivity and Proliferation of *Toxoplasma gondii in vitro*

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Macrophages exposed to 10 µg/mL citrinin (CTR) or 0.01 µg CTR mixed with 0.04 µg aflatoxin B_1 (AFB_1) for a period of 2 h at 37°C, were infected with 10^6 *Toxoplasma gondii* tachyzoites/µL. The parasites were treated with mycotoxins (2 h at 37°C) before being added to the macrophage culture. The number of tachyzoites was quantified 2, 24, 48, 72 and 96 h after infection. During the first 2 hours, 59% infectivity was observed in the control. After exposure to CTR or the mixture of toxins (CTR-AFB_1), macrophages were infected with 77.5% and 75% of the inoculated tachyzoites, respectively. Similarly, 72.3% of the cells were infected when cultured together with previously treated parasites. The treatment with CTR-AFB_1 gave rise to 2.9 times more tachyzoites than the control at 72 h. An increased number of parasites was recovered from macrophages exposed to CTR after 96 h, and to CTR-AFB_1 after 72 h of culture; The number of tachyzoites recovered from the supernatant was 1.94 and 2.06 times higher, respectively, than in the control ($5 \times 10^5 \pm 0.054$ /mL).

Key Words: Aflatoxins, imunossuspression, macrophages, citrinin, *Toxoplasma gondii*.

Inadequately stored products and agricultural by-products exposed to high humidity and high temperatures facilitate the development of fungi. The presence of these microorganisms, in addition to spoiling the products, reduces their quality and favors the development of mycotoxins, which are fungal secondary metabolites. These substances are important, since some are responsible for serious health problems for animals and man. It is known that citrinin, produced by various species of *Penicilium* and *Aspergillus*, when ingested in low concentrations can cause nephropathy in both animals [1,2] and man [3]. The aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are the most powerful hepatocarcinogens found as natural contaminants of food and animal rations [2,4-8]. When ingested in very low concentrations they cause an immunosuppressive effect, leading to a reduction in the natural and acquired resistance to illnesses [9-12]. Mycotoxins are reported to be one of the main causes of outbreaks of coccidioses in domestic animals [13-15]. Since immunosuppressor drugs are of great public health importance, studies on natural Brazilian immunotoxins that are common in the environment are of extreme importance and relevance.

Toxoplasma gondii is an opportunist parasite that affects not only man, but also various species of domestic and wild animals. In immunocompetent individuals, toxoplasmosis usually assumes a benign character, and infection induces a humoral and cellular response that efficiently restricts the pathogenic action, controlling the diffusion of the parasite. In individuals with chronic infection and a compromised immune system, the *Toxoplasma* is freed of the immunological action that curtails it and can invade organs and tissues,

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where it reproduces, causing the serious forms of toxoplasmosis [16,17]. Since *T. gondii* is an intracellular parasite that attacks macrophages, alterations in this host system can can cause antigenic variations, or even alterations in the course of natural infections, which can cause a reactivation of infections in individuals carrying chronic infections [18, 19]. We studied the imunomodulating activity of citrinin (CTR) and its association with aflatoxin B_1 (AFB_1) on macrophages and *T. gondii* tachyzoites, *in vitro*.

Material and Methods

Mycotoxins

Purified and crystallized citrinin (CTR), supplied by the Center of Mycology and Mycotoxicology of the Rural Federal University of Rio de Janeiro, and aflatoxin B_1 (AFB_1) (SIGMA, St. Louis, ME, USA), were solubilized at a ratio of 10 mg/mL of 1M carbonate-bicarbonate buffer solution, pH 9, and were sterilized by filtering through a Millipore Membrane (0.22 μ) into a sterile flask. Solutions containing 10 mg/mL AFB_1 and CTR were diluted before use to a concentration of 100 μg/mL in phosphate buffered saline (PBS). Successive dilutions of these solutions were made to provide final concentrations of 10 μg/mL and 0.01 μg/mL CTR and 0.04 μg/mL AFB_1 per 10^6 cells/mL of cell culture medium.

Animals

Female mice, fed with commercial ration, free of mycotoxins, and with access to drinking water, were supplied by the animal rearing facility of the Rural Federal University of Rio of Janeiro (UFRRJ).

Isolation and culture of macrophages

Six swiss albino mice weighing approximately 20 g were injected intraperitoneally with 0.1 mL/10 g live weight of a 3% Sephadex G-50 suspension in 0.85% saline solution. After 40 h, the mice were sacrificed and their peritoneal cavity washed with 3 mL of solution of 0.3% sodium citrate and this material was then poured into previously cooled tubes. Exudates were centrifuged at 1500 rpm, 10°C for 15 min. The sediment was resuspended in 1 mL of Mit-Glutamin Ohne-$NaHCO_3$ (RPMI 1640) supplemented with 5% fetal calf serum, penicillin (100 U/mL), and streptomycin (50 μg/mL). The viability of the cells was determined by Trypan Blue exclusion [20] in a Newbauer chamber [21]. The macrophages were quantified and kept in suspension at a concentration of 10^6 viable cells/mL in RPMI-1640. Aliquots of 1 mL were placed on cover glasses (5.5 x 22 mm) in sterile Leighton tubes. The tubes were incubated for 48 h at 37°C, 90% humidity and 5% CO_2. After this period, the cell cultures were washed with sterile PBS (pH 7.2) to remove non-adhered cells, and cover glasses with adhered macrophages were used for *in vitro* experiments.

Preparation of inocule for in vitro infection

Tachyzoites were obtained by washing the peritoneal cavities of mice infected with the *T. gondii* (C strain), kindly donated by the Fundação Oswaldo Cruz of Rio de Janeiro (FIOCRUZ), with sterile PBS. Peritoneal washings were centrifuged at 500 rpm, 37°C for 5 min to separate the tachyzoites from the cells. The supernatant was recovered and quantified. Parasite viability was measured by Trypan Blue exclusion. Suspensions containing $1.2x10^6$ tachyzoites/mL were kept under refrigeration until use.

Exposure to mycotoxins

Macrophage cultures were exposed to 10 μg/ml CTR and 0.01 μg/ml CTR associated with 0.04 μg/ml AFB_1 for 2 h at 37°C. Assays in which the tachyzoites (10^6 tachyzoites of *T. gondii*) were previously treated with mycotoxins (association of CTR and AFB_1, 2 h at 37°C) before being added to the cell culture were also performed. Macrophages and tachyzoites not exposed to the mycotoxins were used as controls.

Evaluation of infectivity and proliferation potential of tachyzoites in vitro

The cell cultures were washed with PBS (pH 7.2) before incubation with 1 mL of the tachyzoites suspension (containing $1.2x10^6$) for 2 h at 37°C. The supernatants were then removed and the number of tachyzoites quantified. The macrophage cultures were washed again with PBS (pH 7.2), 1 mL RPMI-1640 added and incubated again at 37°C. This procedure was repeated at intervals of 24, 48, 72 and 96 h after infection, and the number of tachyzoites was scored to determine the relative quantity of parasites delivered to the milieu as a result of its proliferation. The number of intracellular forms of the parasite (infectivity) was estimated by the difference between the median values in the inocule and the tachyzoites delivered per each time point during the course of the experiment.

Statistical analysis of results

Analysis of variance and the Tukey test(estimate of the degrees of freedom as a function of p, Vieira & Hoffman 1989) werer used.

Results

CTR and CTR-AFB_1 repeatedly interfered with the infectivity of the tachyzoites (Table 1). The lowest activity was seen 2 h after infection in the control, in which it was estimated that 59% of the tachyzoites had penetrated the cells. After exposure of macrophages to CTR and to CTR-AFB_1, the tachyzoites percentiles of infection were 77.5% and 75%, respectively. The treatment of infective forms of *T. gondii* with CTR-AFB_1, followed by amendment to macrophage cultures gave rise to the internalization of 72.3% tachyzoites after 2 h of infection. When the parasitic recovery was evaluated in the macrophages exposed to CTR, a significant increase was observed only after 96 h, when 1.94 ($9.7 \times 10^5 \pm 0.07$ tachyzoites/mL) times more tachyzoites were recovered than in the control ($5 \times 10^5 \pm 0.054$ tachyzoites/mL). Macrophages exposed to CTR-AFB_1, started to give increased tachyzoite recovery at 72 h after infection, when $12 \times 10^5 \pm 0.58$ tachyzoites/mL were recovered, which was 2.06 times the number of tachyzoites observed in the control system ($5.8 \times 10^5 \pm 0.18$ tachyzoites/mL) (Figure 1). In this treatment $12 \times 10^5 \pm 0.18$ tachyzoites/mL were recovered, or 2.4 times more parasites than the control ($5 \times 10^5 \pm 0.18$ tachyzoites/mL) at 96 h. Tachyzoites treated with CTR-AFB_1 lead to a greater recovery of parasites during the entire period of the time course experiment. In this case, the most significant results were registered after 72 h of infection, when 2.9 times more tachyzoites were recovered in the cell cultures ($17 \times 10^5 \pm 0.42$ tachyzoites/mL) than was detected in the control ($5.8 \times 10^5 \pm 0.75$ tachyzoites/mL) (Figure 2). The results were closely related, showing a huge reproducibility with a standard deviation never above 0.8 and a significance as large as 99.99%. It demonstrated that a single dose of mycotoxins at concentrations as low as 1 DL50 of CTR or 0.01 DL50 of both CTR and AFB_1 in the mixture CTR- AFB_1, might act on the macrophages, favoring the infectivity and consequent proliferation of *T. gondii*.

Discussion

Macrophages play a crucial role in both non-specific and acquired immune responses. They have a role in the direct destruction of microorganisms [23] and tumoral cells [24,25]. Cell-mediated immunity is the main line of defense against infection by coccidians [26], however the infecting forms of *T. gondii* modify cell functions and the immune response when they penetrate the macrophages, inhibiting the fusion of the lysosomes with vacuoles and, in turn, hindering the action of the degradative enzymes [27]. *T. gondii* grows without alterations inside the macrophages, since this parasite produces large amounts of catalase and peroxidase, which prevent the stimulation of oxidative combustion in macrophages. However, macrophages activated by lymphokines, liberated by sensitized T-cells, interact with the specific antigens of the parasite and acquire the capacity to generate large amounts of hydrogen

Table 1. Table 1 - Evaluation of the infectivity potential of tachyzoites of *Toxoplasma gondii* for peritoneal macrophages, in the different treatments with mycotoxins

Experimental systems	Inocule x 10^6	Tachyzoites x10^5/ 10^6 macrophages	Infectivity %
M	1.2 ± 0.33	7.0 ± 0.38	59
M.C	1.2 ± 0.33	9.3 ± 0.35	77.5
M.C.AFB_1	1.2 ± 0.33	9.0 ± 0.1	75
T.C. AFB_1	1.2 ± 0.33	8.7± 0.2	72.3

Results are the mean of 4 repetitions ($p<0.01$). M- macrophages; M.C.- macrophages exposed to citrinin; M.C.AFB_1- macrophages edposed to the association of citrinin and aflatoxin; T.C.AFB_1- tachyzoites exposed to the association of citrinin and aflatoxin.

Figure 1. Effect of citrinin and its association with aflatoxin B_1 on the proliferation of *Toxoplasma gondii* in a culture of macrophages at different time intervals, where the macrophages were previously exposed to 10 μg/mL CTR and 0.01 μg/mL CTR associated with 0.04 μg/mL AFB_1 for a period of 2 h.

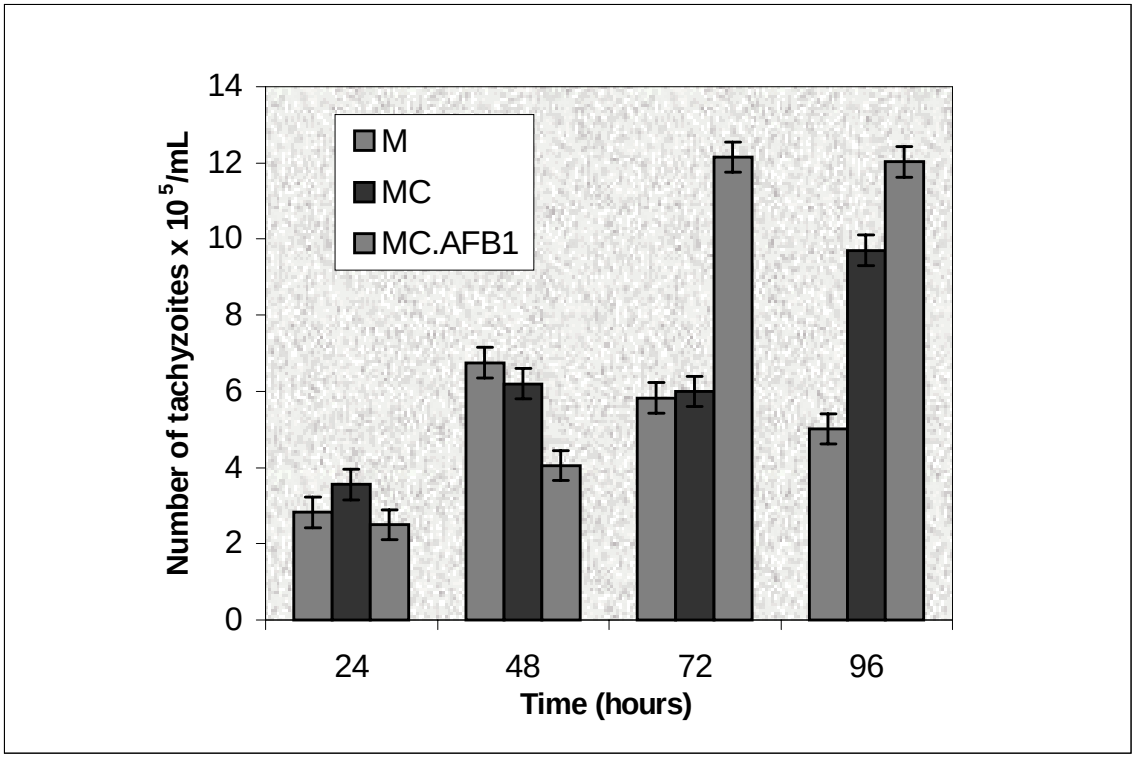
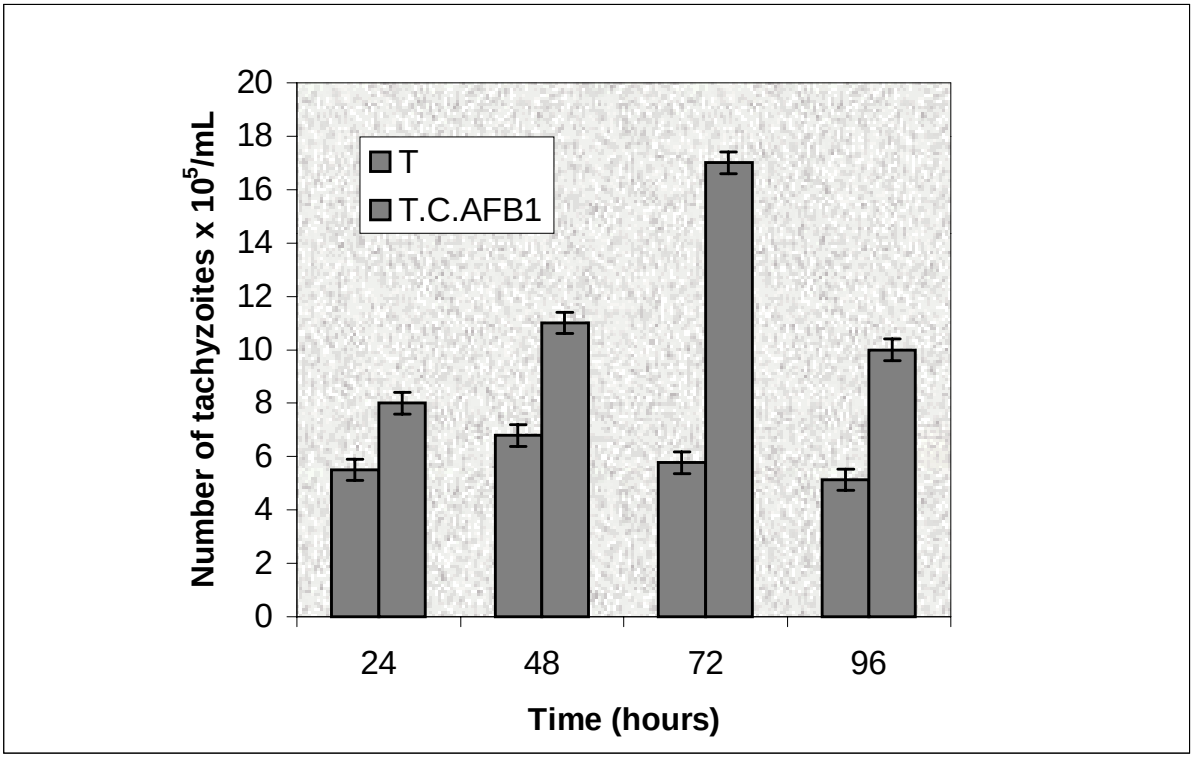


Figure 2. Effect of the association of citrinin and aflatoxin B_1 on the proliferation of the *Toxoplasma gondii* in a culture of macrophages at different time intervals, where the tachyzoites were previously exposed to 0.01 μg/mL of CTR associated with 0.04 μg/mL AFB_1 for a period of 2 h.



peroxide and peroxide ions, promoting powerful inactivation effects against these microrganisms [28].

Some fungal toxins are known to be immunossuppressors; amongst these, AFB_1 is particularly known for its hepatoxic, hepatocarcinogenic and mutagenic effects in man and several other animal species [21,29-31], and CTR is known for its nephrotoxic effects [2,3]. Although the toxic effects of these mycotoxins are known, there is a great lack of data regarding the effects of small concentrations of these toxins. There is a common notion that mycotoxin effects on the immune response might favor the appearance of serious infectious outbreaks [32], or even the reactivation of infections by intracellular parasites, such as *T. gondii* in chronic individuals [16]. Although numerous studies have shown that species, strains, sexes and developmental stages of animals differ in their sensitivity to the effects of toxic chemicals, a clear understanding of the underlying mechanisms is lacking. In fish and wildlife, both innate and differential sensitivities to several toxins are likely to be mediated through a key factor represented by a ligand-activated transcription element. This factor seems to be related to a signal transduction pathway, and it determines sensitivity of species, populations and subpopulations to mycotoxin effects. Similarly, alterations in the signaling of such receptors might be responsible for acquired resistance to mycotoxins [33,34]. The relative sensitivity to several infectious agents may also be somewhat directed by the same kind of key factor that gives rise to the possibility of interactions between naturally occurring toxins and

diseases of high prevalence and morbidity, such as toxoplasmosis.

We evaluated the effect of mycotoxins on the intracellular parasitism of *T. gondii*. In the first series of experiments, the effect of CTR and its association with AFB_1 upon tachyzoite infectivity in cells in culture was evaluated. A significant increase in tachyzoite assimilation by the cells that had been priorly treated with mycotoxins was observed. Such increased assimilation of parasites seems to be directly related to active penetration by a larger number of parasites (Table 1). Previous studies have demonstrated that the cytotoxic action of CTR on macrophages limits the phagocytic processes [1] and that AFB_1 causes significant cytotoxicity in these cells, provoking morphological alterations and causing a reduction in important functions, such as adhesion and phagocytic activity [35, 6], increasing the susceptibility to infectious diseases [10,33,37]. Although the mechanisms by which these mycotoxins exert these effects on the macrophages are not entirely clear, preliminary adhesion of *T. gondii* to the cell's apical complex is known to involve interactions between the parasite and the surface receptors of the target cell [38,39]. Cellular invasion also requires parasite motility, which is dependent upon the extra-cellular pH gradient, which is determined by ions; the internal pH is greater than the external pH [39,40]. The fact that these mycotoxins favor tachyzoite infectivity indicates that they act upon the cellular receptors, increasing the ligation points between the parasites and the cell, facilitating their adhesion, or they may decrease intracellular pH, stimulating the motility of the tachyzoites. When we evaluate the proliferation of the tachyzoites in the cultured macrophages, an increase in tachyzoites in the experimental systems exposed to the mycotoxins was observed. In the cells treated with CTR, there was a significant increase in the proliferation of the tachyzoites, which started after 96 h. *In vitro* studies have demonstrated that CTR has various effects on mitocondrial function and macro-molecule biosynthesis [41], acting on oxidative metabolism [42] and increasing the production of reactive oxygen, in turn, stimulating the production of the superoxide anion in the respiratory chain [43]. The increase in parasitic proliferation in cultured cells exposed to CTR might result from oxidative stress of host cells, which would not display any mechanism of parasite destruction. In cells treated with CTR-AFB_1, a similar increase of parasitic proliferation was observed after 72 h. Such an earlier effect seemed to be related to an addictive toxicity, leading to an increase in host cell mortality. Previous studies have demonstrated that the effect of the combination of mycotoxins affects immunocompetent cells [44], being able to significantly increase toxicity to myelocytes [34]. The recovery of integrally viable parasites after direct treatment with mycotoxins, leading to an increased recovery of parasites delivered by the host cells during the time course experiment indicated that the toxic chemicals interact with tachyzoites trough distinct mechanisms. The similarity in the parasite recovery rate in the two systems of cells-toxins and parasites-toxins might not be related to any ordinary mechanism. One possibility would be the formation of surface complexes that facilitate the host parasite interaction, since a tendency to form cellular agglutinates was observed when in the presence of these mycotoxins (data not shown). This possibility is reinforced by the fact that the entire cellular system exposed to mycotoxins was washed three times by centrifugation (600 x g/10 min/4 ± 1°C) with mycotoxin-free RPMI prior to the infection experiments and that all inoculations were carried out with previously quantified live infective forms. The combined results might also be related to the decrease in the primary functions of the macrophages, as previously demonstrated for AFB_1 [35,36]. Macrophages, when activated, increase their phagocytic activity and liberate products, such as cytokines and intermediate reagents for non-specific primary defense against infectious agents [17, 19]. AFB_1 modifies the functions of the macrophage, decreasing the secretion of IL-1 and IL-6 and the production of TNF-α, nitric oxide, superoxide anion and hydrogen peroxide [36]. Nevertheless, a significant increase in tachyzoites in the experimental systems exposed to the association of CTR and AFB_1 was related to a primary contact and did not indicate

any kind of memory inhibition of the parasites, but rather a direct host cell cytotoxic effect. Otherwise, a reduction of IL-6, TNF and nitric oxide, which are important in the control of tachyzoite replication in the acute phase of the infection, should also be expected in long-term infections in individuals exposed to low doses of common, naturally occurring mycotoxins. In our experimental model a plateau of recovery was observed following emission of viable parasites. This could be related to limitations of the experimental system itself. The diminution of viable target host cells increased with time, and there was a significant diminution in the capabilities of capture and metabolism for the proliferation of the parasites. These data indicate that there is selection by apoptosis; further investigations are required to clarify the possible mechanisms of the actions of these mycotoxins on *T. gondii*. Unfortunately specific anti-metabolites, which could be used to isolate the pathways involved in parasitism, are still unknown. Due to the easy reproducibility of our experimental model, it may provide comparative results, which should be useful to better understand this modality of environmental interaction. Cultures of macrophages from chronically infected individuals, as well as from acute and sub clinical infections would be of great interest. The possibilities of studying the genetic expressions of chemokines, as well as the mechanisms of immunity and genetic sensitivity to different environmental toxicants are also of great importance.

Our results reinforce the suggestion that mycotoxins, even at low concentrations, act on tachyzoites and macrophages to favor the infectivity and proliferation of *T. gondii*, and that association with these mycotoxins enhances pathologies to the immune cells. It also appears clear that increased monitoring and control, as well as revision of the legal acceptable levels for these toxins, is necessary, since in Brazil, despite the current legislation, there aflatoxins are abundant, and there is a high level of incidence in foods used for human and animal consumption, such as maize, peanuts and their derivatives, putting immune compromised individuals, such as children and those who have AIDS, at risk, especially in the rural areas of the country.

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