

Opportunistic infection by *Toxoplasma gondii* caused by exposure to coumarins originated from toxigenic fungi

Infecção oportunista por *Toxoplasma gondii* causada pela exposição a micotoxinas cumarínicas

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SUMMARY – Macrophages exposed to 10 μ g citrinin (CTR) or 0.01 μ g CTR mixed to 0.04 μ g aflatoxin B₁ (AFB₁) for a period of 2h at 37°C, were infected with 10⁶ *Toxoplasma gondii* tachyzoites/ml. Parasites were also previously treated with mycotoxins (2h at 37°C) before being added to the macrophage culture. The number of tachyzoites was quantified 2, 24, 48, 72 and 96h after infection. During the first 2 hours, 59% infectivity was observed in the control. After exposure to CTR or the mixture of toxins (CTR-AFB₁) 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 protozoan treatment with CTR-AFB₁ gave rise to a tachyzoites pick number 2.9 times higher than the control at 72h. It was recovered an increased number of parasites from macrophages exposed to CTR after 96h, as well as with CTR-AFB₁ after 72h of culture, when tachyzoites recovered from the supernatant were respectively 1.94 (9.7x10⁵±0.07 tachyzoites/ml) and 2.06 (12x10⁵±0.58 tachyzoites/ml) times higher than in the control (5x10⁵±0.054 tachyzoites/ml).

KEYWORDS – Aflatoxins; immunosuppression; macrophages; citrinin; *Toxoplasma gondii*.

RESUMO – Macrófagos expostos a 10 μ g de Citrinina (CTR) ou a 0,01 μ g de CTR misturados a 0,04 μ g de Aflatoxina B₁ (AFB₁), por um período de 2h a 37°C, foram infectados com um inoculo de 10⁶ taquizoítas/ml de *Toxoplasma gondii*. Os parasitos foram previamente tratados com as micotoxinas (2h a 37°C) antes de serem adicionados a cultura de macrófagos. O número de taquizoítas foi determinado 2, 24, 48, 72 e 96h após a infecção. Após as primeiras 2 horas, 59% de infectividade foi observado no grupo controle. Após a exposição a CTR ou a mistura de toxinas (CTR-AFB₁), os macrófagos foram infectados com 77,5% e 75% dos taquizoítas inoculados, respectivamente. Do mesmo modo, 72,3% das células foram infectadas quando cultivadas com os parasitos previamente tratados com as micotoxinas. O tratamento dos parasitos com a mistura CTR-AFB₁ promoveu um aumento de 2,9 vezes na contagem de taquizoítas, em relação ao grupo controle, após 72h. Um maior número de parasitos foi recuperado da cultura de macrófagos exposta a CTR, após 96h de cultivo, e da exposta à CTR-AFB₁, após 72h de cultivo, sendo que a quantidade de taquizoítas recuperada do sobrenadante foi, respectivamente, 1,94 (9,7x10⁵±0,07 taquizoítas/ml) e 2,06 (12x10⁵±0,58 taquizoítas/ml) vezes maior que no grupo controle (5x10⁵±0,054 taquizoítas/ml).

PALAVRAS-CHAVE – Aflatoxina, imunossupressão, macrófagos, citrinina, *Toxoplasma gondii*.

INTRODUÇÃO

Inadequately stored products and agricultural by products exposed to humidity and high temperatures facilitate the development of fungi. The presence of these microorganisms, in addition to spoiling the products, reduce its 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 *Penicillium* and *Aspergillus*, when ingested in low concentrations might cause nephropathy in both, animals (Hald, 1991) and in man (Castegnaro et al., 1990). The aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are the most powerful hepatocarcinogens found as natural contaminants of food and rations (Robens & Richards, 1992). When ingested in very low concentrations they cause an immunosuppressive effect, leading to a reduction in the natural and acquired resistance to illnesses (Sharma, 1993). Mycotoxins are reported to be one of the main causes of outbreaks of coccidiosis in production ani-

mals (Smith & Moss, 1985). Since immunosuppressor drugs are of great public health importance, studies concerned to natural Brazilian immunotoxins of increased environmental prevalence are of extreme importance and relevance.

Toxoplasma gondii is an opportunist parasite that affects not only man, but also diverse species of domestic and wild animals. In immunocompetent individuals, toxoplasmosis usually assumes a benign character and infection induces an humoral and cellular response that efficiently restricts the pathogenic action, controlling the diffusion of the parasite. In individuals with chronic infection, with a compromised immune system, the *Toxoplasma* is freed of the immunological action that curtails it and can invade organs and tissues where it reproduces, causing the serious forms of toxoplasmosis (Luft & Remington, 1992). Since *T. gondii* is an intracellular parasite which utilizes macrophages, alterations in this host system can determine antigenic variations, or even alterations in the course of natural infections, which can cause the reactivation of infections in individuals carrying chronic infections (Venturini et al., 1996). The present study aimed to establish

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the extent of the immunomodulating activity of citrinin (CTR) and its association with aflatoxin B₁ (AFB₁) on macrophages and *Toxoplasma gondii* tachyzoites, in vitro, before and after poisoning.

MATERIAL AND METHODS

Mycotoxins: purified and crystallized citrinin (CTR), supplied by the Center of Mycology and Mycotoxicology at the Rural Federal University of Rio de Janeiro (UFRRJ), and aflatoxin B₁ (AFB₁) (SIGMA, St. Louis, ME, USA), were solubilized in a ratio of 10mg/ml of solution 1M carbonate-bicarbonate buffer, pH 9, and were sterilized by filtering through a Millipore Membrane (0,22mm) into a sterile flask. Solutions containing 10 mg/ml AFB₁ and CTR were diluted before use to a concentration of 100mmg/ml in phosphate buffered saline (PBS). Successive dilutions of these solutions were made to provide final concentrations of 10mmg/ml and 0,01mmg/ml CTR and 0,04mmg/ml AFB₁ per 10⁶ cells/ml of cell culture medium.

Animals: female mice, fed with commercial ration free of mycotoxins, and given drinking water were supplied by the animal house of the Rural Federal University of Rio de Janeiro (UFRRJ).

Isolation and culture of macrophages: six swiss albino mice weighing approximately 20g were injected intraperitoneally with 0,1ml/10g live weight of a 3% Sephadex G-50 suspension in 0.85% saline solution. After 40h, the mice were sacrificed and their peritoneal cavity washed with 3 ml of solution of 0.3% sodium citrate and poured into previously cooled tubes. Exudates were centrifuged at 1500rpm, 10°C for 15min. The sediment was resuspended in 1ml of Mit-Glutamin Ohne-NaHCO₃ (RPMI 1640) supplemented with 5% fetal calf serum, penicillin (100U/ml), and streptomycin (50mmg/ml). The viability of the cells was determined by Trypan Blue exclusion (Phillips, 1973) in a Neubauer chamber (Qureshi & Hagler, 1992). The macrophages were quantified and kept in suspension at a concentration of 10⁶ viable cells/ml in RPMI-1640. Aliquots of 1ml were placed on cover glasses (5.5x22mm) in sterile Leighton tubes. The tubes were incubated for 48h at 37°C, 90% humidity and 5% CO₂. 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 inocula 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 Oswaldo Cruz Foundation of Rio de Janeiro (FIOCRUZ), with sterile PBS. Peritoneal washings were centrifuged at 500 rpm, 37°C for 5min to separate the tachyzoites from the cells. The supernatant was recovered and quantified. Parasite viability was measured by Trypan Blue exclusion. Suspensions containing 1.2x10⁶ tachyzoites/ml were kept under refrigeration until use.

Exposure to mycotoxins: Macrophage cultures were exposed to 10⁶g CTR and 0.01g CTR associated with 0.04 g AFB₁ for 2h at 37°C. Assays in which the tachyzoites (10⁶ tachyzoites of *T gondii*) were previously treated with mycotoxins (association of CTR and AFB₁, 2h 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 1ml of the tachyzoite suspension (containing 1.2x10⁶) for 2h 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), added of 1ml RPMI-1640 and incubated again at 37°C. This procedure was repeated at the intervals of 24, 48, 72 and 96h after infection and, the number of tachyzoites scored to determine the relative quantity of parasites delivered to the milieu as a result of their proliferation. The number intracellular forms of parasite (infectivity) was estimated by the difference between the median values related to the inocula and the delivered tachyzoites per each point during the time course experiment.

Statistical analysis of results: the validity of the results was verified on the basis of the analysis of variance and agreement of Tukey (estimate of the degrees of freedom in function of p) (Vieira & Hoffman, 1989).

RESULTS

CTR and CTR-AFB₁ repeatedly interfered with the infectivity of the tachyzoites (Table I). The lowest activity was seen 2h after infection in the control system in which it was estimated that 59% of the tachyzoites had penetrated the cells. Otherwise, after exposure of macrophages to CTR and to CTR-AFB₁, the tachyzoites percentiles of infection were of 77.5% and 75%, respectively. The treatment of infective forms of *T gondii* with CTR-AFB₁ followed by amendment to macrophages cultures had given rise to the internalization of 72.3% tachyzoites after 2h of infection. When the parasitic recovering was evaluated in the macrophages exposed to CTR, a significant increase was observed only after 96h, when it was recovered 1.94 (9.7x10⁵±0.07 tachyzoites/ml) times more tachyzoites than in the control system (5x10⁵±0.054 tachyzoites/ml). Macrophages exposed to CTR-AFB₁, started to rise the tachyzoites recovering at 72h after infection, when it was recovered 12x10⁵±0.58 tachyzoites/ml, which represented 2.06 times the tachyzoites number observed in the control system (5.8x10⁵±0.18 tachyzoites/ml) (Fig 1). In this treatment was recovered 12x10⁵±0.18 tachyzoites/ml, or 2.4 more parasites than the control (5x10⁵±0.18 tachyzoites/ml) at 96h. Tachyzoites treated with CTR-AFB₁, lead to a greater recovering of parasites during all the period in which the time course experiment was carried

TABLE I
Evaluation of the infectivity potential of tachyzoites of *Toxoplasma gondii* in peritoneal macrophages according to the different treatments with mycotoxins - Results are the mean of 4 repetitions (p<0.01)

Experimental systems	Inocule Initial x 10 ⁶	Tachyzoites x 10 ⁵ /10 ⁶ macrophages	Percentage of 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%

M. macrophages; M.C.- macrophages exposed to citrinin; M.C.AFB₁- macrophages exposed to the association of citrinin and aflatoxin; T.C.AFB₁- tachyzoites exposed to the association of citrinin and aflatoxin.

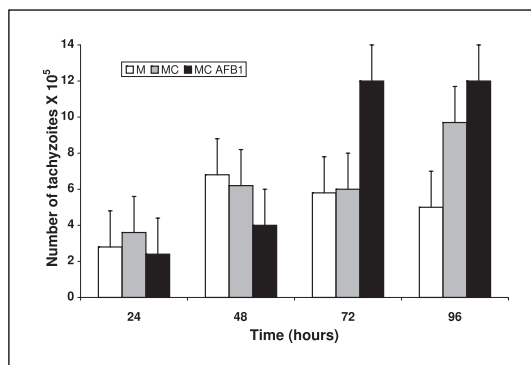


FIG. 1 - Effect of citrinin and its association with aflatoxin B₁ on the proliferation of the *Toxoplasma gondii* in a culture of macrophages at different time intervals, where the macrophages were previously exposed to 10µg CTR and 0.01µg CTR, associated with 0.04µg AFB₁ for a period of 2h.

out. In this case, the most significant results were registered after 72h of infection, when it was recovered 2.9 times more tachyzoites in the cell cultures ($17 \times 10^5 \pm 0.42$ tachyzoites/ml) than it was detected in the control ($5.8 \times 10^5 \pm 0.75$ tachyzoites/ml) (Fig. 2). Results were closely related, showing a huge reproducibility with standard deviation never superior to 0.8 and significance as big as of 99.99%. It served to demonstrate that a single dose of mycotoxins at concentrations as low as 1 DL50 of CTR or 0.01 DL50 of both CTR and AFB₁ in the mixture CTR-AFB₁, 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 (Macmicking et al., 1997) and tumoral cells (Qureshi & Miller, 1991; Chang et al., 2001). The immunity mediated by cells is the main line of defense against infection by coccidia (Lillehoj & Trout, 1994), however the infecting forms of *T. gondii* modify cell functions and the immune response when penetrating the macrophages, inhibiting the fusion of the lysosomes with vacuoles and, in turn, hindering the action of the degradative enzymes (Sibley & Boothdoyds, 1991). *T. gondii* grows without alterations inside the macrophages, since they contain large amounts of catalase and peroxidase which prevent the stimulation of macrophagic oxidative combustion. 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 peroxide and peroxide ions, acquiring powerful inactivation functions against these microorganisms (Krahenbuhl & Remington, 1980).

Some fungal toxins are known to be immunosuppressors, amongst these, AFB₁ is particularly known for its hepatotoxic, hepatocarcinogenic and mutagenic effects in man and several other animal species (Qureshi & Hagler, 1999; Sahoo et al., 2001), and CTR is known for its nephrotoxic effect (Fink-Gremmels, 1999; Pitt, 2000). 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 or the effects of their associations

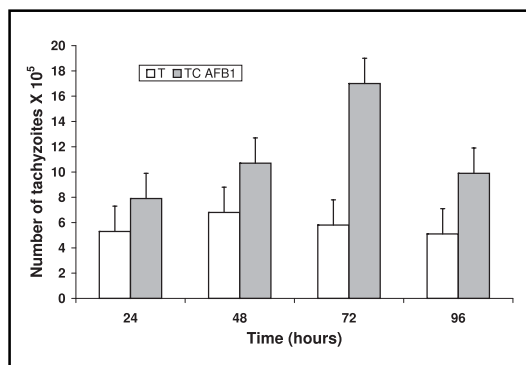


FIG. 2 - Effect of the association of citrinin and aflatoxin B₁ on the proliferation of the *Toxoplasma gondii* in a culture of macrophages at different time intervals, where the tachyzoites were previously exposed to 0.01µg of CTR associated with 0.04µg AFB₁ for a period of 2h.

upon the immune response, which could favor the appearance of serious infectious outbreaks (Schuch, 1989), or even the reactivation of infections by intracellular parasites, such as *T. gondii* in chronic individuals (Luft & Remington, 1992). Although numerous studies have shown that species, strains, sexes and developmental stages of animals differ in their sensitivity to 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.

Such factor seems to be related to a signal transduction pathway and determine sensitivity of species, populations and subpopulations to mycotoxins effects. Similarly, alterations in such receptors signaling might be responsible for acquired mycotoxins resistance (Pier et al., 1980). The relative sensitivity to several infectious agents may be also somewhat directed by the same kind of key factor which gives rise to the possibility of interactions between naturally occurring toxins and diseases of high prevalence and morbidity such as toxoplasmosis.

In the present study, the effect of mycotoxins on the intracellular parasitism of *T. gondii* was evaluated. In the first series of experiments, the effect of CTR and its association with AFB₁ upon tachyzoite infectivity in cells in culture was evaluated. It was observed a significant augmentation of tachyzoites assimilation by the cells treated at prior with mycotoxins. Such increased assimilation of parasites seemed to be directly related to an active penetration by a larger number of parasites (Table I). Previous studies have demonstrated that the cytotoxic action of CTR on macrophages limits the phagocytic processes (Frank, 1992) and that AFB₁ causes significant cytotoxicity in these cells, provoking morphologic alterations and causing a drawback on important functions such as adhesion and phagocytic activity (Neldon-Ortiz & Qureshi, 1992), increasing the susceptibility to infectious diseases (Pier et al., 1980). Although the mechanisms by which these mycotoxins exert these effects on the macrophages are not entirely clear, preliminary adhesion of the *T. gondii* by the cell's apical complex is known to involve interactions between the parasite and the surface receptors of the target cell (Minco & Kasper, 1994). Cellular invasion also requires parasite mo-

tility, which is dependent upon the extra cellular pH gradient which is determined by ions, where the internal pH is greater than the external pH (Endo & Yagita, 1990). The fact that these mycotoxins favor tachyzoite infectivity, indicates that they act upon the cellular receptors, increasing the ligation points between the parasite and the cell, facilitating its adhesion, or that they may decrease intracellular pH, stimulating the motility of the tachyzoites. When evaluating 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 96h. In vitro studies demonstrate that CTR has various effects on mitochondrial function and macro-molecule biosynthesis (Braumberg et al., 1992), acting on the oxidative metabolism (Chagas et al., 1995) and increasing the production of reactive oxygen, in turn, stimulating the production of the superoxide anion in the respiratory chain (Ribeiro et al., 1997). The increase in the parasitic proliferation in cultured cells exposed to CTR might result of oxidative stress of host cells which could not display any mechanism of parasite destruction. Cells treated with the CTR-AFB₁, a similar increase of the parasitic proliferation was observed after 72h. Such earlier effect seemed to be related to an addictive toxicity leading to the augmentation of host cells death. Previous studies have demonstrated that the effect of the combination of mycotoxins should affect immunocompetent cells, being able to significantly increase the toxicity to myelocytes (Terse et al., 1993). The recovering of integrally viable parasites after direct treatment with mycotoxins leading to an increasing recovering of parasites delivered by the host cells during the time course experiment suggested the toxic chemicals interact with tachyzoites through distinct mechanisms. The similarity between the parasite recovering in both systems cells-toxins and parasites-toxins might not be related to any mechanism somewhat ordinary. A possibility should be the formation of surface complexes that facilitate the host parasite interaction, since it was observed a tendency to form cellular agglutinates when in the presence of these mycotoxins (data not shown). This possibility is enforced by the fact that all cellular system exposed to mycotoxins was washed three times by centrifugation (600xg/10min/4 ± 1°C) with mycotoxins-free RPMI prior the infection experiments and that all inoculation was carried out with previously quantified live infective forms. The combined results might also be related to the reduction of the macrophages primary functions, as previously demonstrated for AFB₁ (Neldon-Ortiz & Qureshi, 1992). Macrophages, when activated, augment their phagocytic activity and liberate products such as cytokines and intermediate reagents for non-specific primary defense against infectious agents (Alexander et al., 1997). AFB₁ 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 (Moon et al., 1999). Despite of that, a significant increase in tachyzoites in the experimental systems exposed to the association of CTR and AFB₁, was related to a primary contact and did not represent any kind of memory inhibition of the parasites but a direct host cell cytotoxic effect.

Otherwise, a reduction of IL-6, TNF and nitric oxide, which are important in the control of the tachyzoite replication in the acute phase of the infection, should also be expected for long-term infections in individuals exposed to low doses of natural occurring mycotoxins of high environmental occurrence. In the present experimental model it was observed the presence of a plateau of recovering following the pick of viable parasites delivering. It should be related to limitations of the experimental system itself. The diminution of viable target host cells was affected with the time and suffered a significant diminution in its capabilities of capture and metabolism for the proliferation of the parasites. These data suggest a selection by apoptosis and further investigations are required to clarify the possible mechanisms of the actions of these mycotoxins on *T gondii*. Unfortunately specific anti-metabolites, which should be used in order to isolate the pathways, which should be related to favor the parasitism, are still unknown. Due to the great reproducibility of the experimental model used, it may be concluded it represents an excellent source of comparative results, which should be useful to highlight this modality of environmental interaction. Cultures of macrophages from chronically infected individuals, as well as from acute and sub clinical infections should be of great interest. The possibilities of studying their genetic expressions of chemokines, as well as the mechanisms of immunity and genetic sensitivity to different environmental toxicants are also of great importance.

The results presently obtained reinforced the suggestion mycotoxins, even at solely exposure and low concentration, act on the tachyzoites and macrophages to favor the infectivity and proliferation of the *T gondii* and that the association of these mycotoxins enhances pathologies on the immune cells. It may also be of importance to point out that greater monitoring and control, as well as the revision of the legal levels acceptable for these toxins, is necessary, since in Brazil, despite the current legislation, there is a huge aflatoxin occurrence and a high level incidence in foods used for human and animal consumption, such as maize, peanuts and their derivatives putting at risk immune compromised individuals, such as children and those who got AIDS, specially at the rural areas of the country.

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