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In-Vitro Cytotoxicity of Aflatoxin B_1 to Broiler Lymphocytes of Broiler Chickens

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■Keywords

Aflatoxin B1; Broilers; Lymphocytes; PicoGreen; Reactive oxygen species.

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ABSTRACT

The aim of the present work was to study the *in-vitro* cytotoxic effects of different concentrations of aflatoxin B_1 (AFB_1) on broiler lymphocytes. Lymphocyte-rich mononuclear cells were separated by Ficoll-Histopaque density and cultured in 96-wellplates containing the evaluated AFB_1 concentrations in 5% CO_2 atmosphere at 39°C. Thereafter, MTT, PicoGreen, and reactive oxygen species assays were performed. Cell viability decreased in the presence of 10 µg/mL AFB_1 at 48 h ($p < 0.05$) and of 10 and 20 µg/mL AFB_1 at 72 h ($p < 0.01$ and $p < 0.001$, respectively) when compared to the control (0 µg/mL). However, a dose-dependent increase in the cell-free DNA at 24 h was observed at 1, 10 and 20 µg/mL ($p < 0.001$). ROS formation significantly increased at 24 h at all concentrations ($p < 0.001$). The *in-vitro* results demonstrate that AFB_1 is cytotoxic and causes biomolecular oxidative damage in broiler lymphocytes.

INTRODUCTION

Aflatoxins are secondary metabolites of *Aspergillus* fungi, including *A. flavus*, *A. parasiticus,* and *A. nomius* (Hamid *et al.*, 2013; Kalpana *et al*., 2012). The most common aflatoxins are B_1, B_2, G_1 and G_2 that are naturally present in many food products, and M_1 and M_2 are found in milk, dairy products, eggs, meat, and urine (Oliveira *et al*., 2000).

The toxigenic potential of aflatoxins dependends on their dose and duration of intake, as well as on animal species, age, and nutritional status (Marai & Asker, 2008). It is directly associated with its rapid absorption in the gastrointestinal tract and immediate binding to serum proteins, such as albumin (Santurio, 2000). AFB_1 is recognized as one of the most potent known liver carcinogens, and has genotoxic, immunotoxic, and other adverse effects on several animal species, including poultry (Kalpana *et al*., 2012).

In addition of its high mutagenic and teratogenic potential, the constant daily intake of small amounts of AFB_1 is responsible for the induction and modulation of diseases in humans and animals (Lewis *et al*., 1999). AFB_1 mainly affects cell-mediated immunity (Williams *et al*., 2004), reducing lymphocyte proliferation and cytokine production in experimental animals (Abbès *et al*., 2010). Moreover, AFB_1 is able to generate reactive oxygen species (ROS), which may have a dual role, acting as toxic bioproducts that alter the cellular function and viability and as key participants in cell regulation and signaling (Shen *et al*., 1996). The carcinogenic action of AFB_1 requires its metabolic activation by cytochrome (CYP) P-450, which is primarily responsible for the activation of AFB_1 to produce the ultimate carcinogen AFB_1-8,9-epoxide (Marai & Asker, 2008), and binds to DNA and RNA (Bbosa *et al*., 2013). DNA damage also occurs when ROS synthesis exceeds the capacity of

body antioxidant defenses to eliminate them (Bischoff & Ramaiah, 2007).

Chronic AFB_1 exposure causes significant losses in the poultry industry (Sur & Celik, 2003). Contamination with AFB_1 can negatively affect broiler chickens in a variety of ways, including reduced fitness, altered immune function (Yunus *et al*., 2011), decreased survival (Azzam & Gabal, 1998), and reduced humoral immune response to vaccines (Gabal & Azzam, 1998).

Based on this context, the aim of the present study was to use MTT, PicoGreen fluorescence and the 2',7' dichlorofluorescein diacetate (DCFH-DA) assays to investigate the effects of AFB_1 on lymphoid cells of broiler chickens cultivated *in vitro*.

MATERIALS AND METHODS

Chemicals

AFB_1 ($C_{17}H_{12}O_6$ - 5mg), RPMI-1640 medium, Ficoll-Histopaque density®, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium bicarbonate, penicillin/streptomycin solution and DCFH-DA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum was obtained from Cultilab (Campinas, SP, BR), Vybrant® MTT cell Proliferation Assay Kit and Quant-iT™ PicoGreen® Reagent from Invitrogen (Eugene, UK).

Birds

Broiler chickens (*Gallus gallus domesticus)* between 38 and 42 days of age and weighing 2-2.5 kg were used in the experiments. Birds were obtained from a local processing plant, after being stunned by electronarcosis, and bled by sectioning the large vessels of the neck. All experimental procedures were conducted according to Normative Instruction N° 3, January 17th, 2000 (Regulation of Technical Methods for the Humane Slaughter of Animals; Brasil, 2000).

AFB_1

AFB_1 (5 mg - Sigma-Aldrich Co.) was first dissolved in 99% ethanol (Mehrzad *et al.*, 2011). Further dilutions were made with RPMI 1640 complete medium containing 9.7 mM HEPES and 24 mM sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum and 2.5 IU penicillin/streptomycin. AFB_1 was added to the medium containing the isolated lymphocytes at the final concentrations of 0.1, 1, 10, and 20 µg/mL with 0.5 % v/v of ethanol in the culture cell. Vehicle control cells were prepared in the same manner as AFB_1 treated samples, including the addition of the vehicle (0.5% ethanol) instead of AFB_1.

Peripheral blood mononuclear cell preparation

Lymphocyte-rich mononuclear cells of broiler chickens were isolated from blood collected with 7.2 mg dipotassium EDTA and separated on Ficoll-Histopaque density system (Sigma-Aldrich Co.) as described by Böyum (1968) and Nathanson (1982), except that lymphocytes were centrifuged at 1000 rpm for 5 min with culture medium to completely remove platelets. Next, the pellet was suspended in culture medium and stored in a culture flask in a 5% CO_2 atmosphere at 39° C for 2 h to allow adhesion of monocytes to the surface of the bottle, which times occur among lymphocytes (non adherent). After incubation, cell suspensions were transferred to centrifuge tubes and centrifuged at 1500 rpm for 10 min. The supernatants were discarded, the dry pellets containing lymphocytes were suspended in 3 mL of culture medium, and cell viability was measured using trypan blue dye (1:2). This protocol was performed within 2 h following blood collection.

Lymphocytes were suspended at a density of 0.7 x 10^5 cells/mL in RPMI 1640-enriched culture complete medium. Cells were seeded in triplicate in 96-well tissue culture plates under an atmosphere of 5% CO_2 at 39°C, and treated with increasing concentrations of AFB_1 (0, 0.1, 1, 10 and 20 µg/mL). The density of the cells that were seeded was equivalent to 75% confluence.

MTT assay

Cytotoxicity was evaluated by MTT reduction assay (Vybrant MTT cell Proliferation Assay Kit), which is based on the cleavage of tetrazolium salts via the activity of mitochondrial succinate dehydrogenase in metabolically active cells that yield a colored formazan product (Mosmann, 1983). Since the conversion takes place in living cells, the amount of formazan produced directly corresponds to the number of viable cells. Absorbance was measured in microplate spectrophotometer at 540 nm. This assay was performed 24, 48 and 72 h after AFB_1 exposure. All tests were performed in 96-well microplates in triplicate. The results were expressed as optical density (OD_{540}).

PicoGreen fluorescence assay

The PicoGreen fluorescence assay measures the presence of double-stranded (ds) DNA, which is an indicative of cytotoxicity (Swarup *et al*., 2011). This assay was performed 24, 48 and 72 h after exposure, according to the protocol supplied by the manufacturer (Quant ItTM, Invitrogen). PicoGreen dye was diluted to

Table 1 – Cell viability of broiler chicken lymphocytes incubated for 24, 48 and 72 h at increasing concentrations of AFB1. Data are expressed as means ± standard deviations of the optical densities recorded at 540 nm.

Time	AFB_1 concentration (µg/ml)				
	0	0.1	1	10	20
0 h	0.512 ± 0.02	0.514 ± 0.01	0.511 ± 0.02	0.509 ± 0.03	0.507 ± 0.03
24 h	0.514 ± 0.01	0.500 ± 0.01	0.520 ± 0.01	0.509 ± 0.03	0.507 ± 0.01
48 h	0.761 ± 0.02	0.768 ± 0.03	0.748 ± 0.02	0.721 ± 0.03*	0.785 ± 0.02
72 h	0.760 ± 0.01	0.776 ± 0.01	0.782 ± 0.02	0.696 ± 0.04***	0.705 ± 0.01**
Time effect	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, compared to control group (0 µg/ml AFB_1) within the time of exposure.

1:200 with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and incubated with each supernatant sample in the dark at room temperature in black 96-well microplates for 5 min. All fluorescence measurements were recorded with a fluorimeter. Fluorescence emissions of PicoGreen alone (blank) and PicoGreen with supernatant were recorded at 520 nm using an excitation wavelength of 480 nm at 25°C. A standard curve was generated using the lambda DNA standard provided by the manufacturer. All calibration samples were assayed in quintuplicate. Baseline fluorescence was determined with a TE blank, which average was subtracted from the average fluorescence of the other samples. The results were expressed as fluorescence (pg/mL of dsDNA).

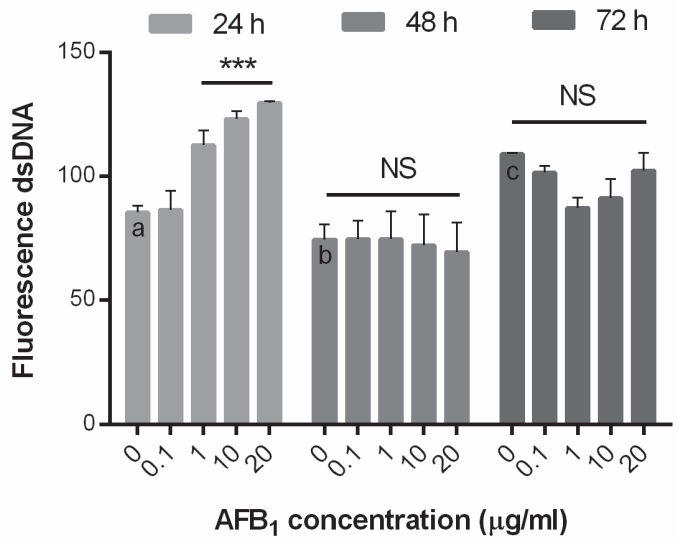

Figure 1. Free dsDNA quantification. Lymphocytes of broiler chickens were exposed to increasing concentrations of AFB1 at 24, 48 and 72 h and dsDNA levels were measured by the PicoGreen assay. Data are means ± standard deviations. ***$p < 0.001$ compared to the control group (0 µg/ml AFB1) at each time point. Letters indicate significant differences among time points for the group without AFB1 ($p < 0.05$).

Determination of intracellular ROS formation

Intracellular ROS concentrations were determined using the fluorescence probe DCFH-DA, which is a well-established compound used to detect and quantify free radicals, particularly intracellular hydrogen peroxide (H_2O_2). DCFH-DA is transported across the cell membrane and it is deacetylated by cytosolic esterases to form non-fluorescent DCFH, which is trapped within the cells. DCFH is converted to fluorescent DCF by the action of peroxide rated by the presence of peroxidase (Halliwell & Whiteman, 2004; LeBel *et al*., 1992).

After each time point of exposure (24, 48 and 72 h), cells were treated with DCFH-DA (10 µM) for 60 min at 37°C. Fluorescence was measured at 488 nm excitation and 525 nm emission. All tests were performed in 96-well microplates, in quintuplicate for each of the samples that were tested, and the results were expressed as fluorescence intensity (nm).

Statistical analysis

The data were normally distributed. Therefore, data within each time point were submitted to one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. ANOVA followed by Tukey's test was used to calculate overall time effects. The results with $p < 0.05$ were considered significant. The data were pooled from three independent experiments, and the results were expressed as the mean and standard deviation.

RESULTS

MTT assay

The lymphocyte viability of broiler chickens was assessed in the presence of AFB_1 using the MTT assay at 24, 48, and 72 h (Table 1). Compared with the control (0 µg/mL), this mycotoxin induced significant decrease of cell viability at the concentration of 10 µg/mL ($p < 0.05$) at 48 h, and at the concentrations of 10 and 20 µg/mL ($p < 0.001$) at 72 h of incubation.

PicoGreen fluorescent assay

The effects of AFB_1 on free dsDNA in lymphocytes of broiler chickens were evaluated using the PicoGreen

fluorescent assay (Fig. 1). An increase in dsDNA was observed at the AFB_1 concentrations of 1, 10, and 20 µg/mL at 24 h ($p < 0.001$). No significant differences from the control group (0 µg/mL) were observed at 48 and 72 h.

DCFH-DA assay

Intracellular ROS formation was examined using a fluorescence sensitive probe, through the DCFH-DA assay. When compared with the control (0 µg/mL), an increase in ROS concentrations was observed at 24 h of exposure to AFB_1 at the concentrations of 0.1, 1, 10, and 20 µg/mL ($p < 0.001$) (Fig. 2). At 48 and 72 h, the formation of ROS in cultures of broiler chicken lymphocytes tended to stabilize.

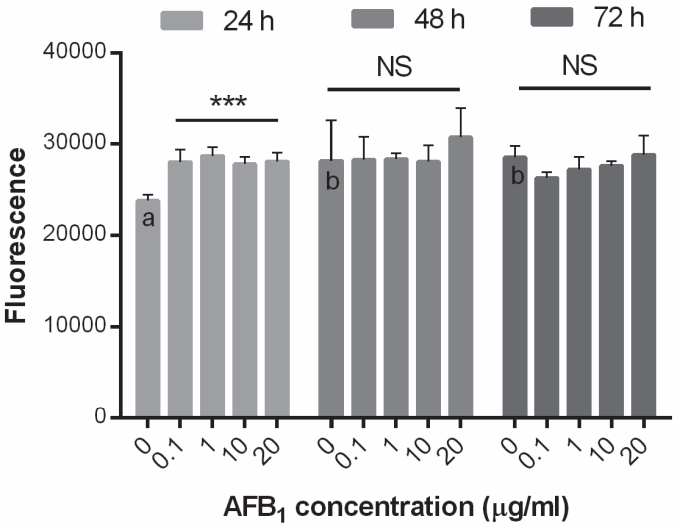

Figure 2. Intracellular formation of ROS measured by the DCFH-DA assay. Lymphocytes of broiler chicken were exposed to increasing concentrations of AFB1 and the fluorescence intensity was monitored at 24, 48 and 72 h of incubation. Data are means ± standard deviations. ***$p < 0.001$ compared to the control group (0 µg/ml AFB1) at each time point. Letters indicate significant differences among time points for the group without AFB1 ($p < 0.01$).

DISCUSSION

Immune function is a complex process comprising different elements of the immune system that must work together to elicit an effective immune response. Therefore, the ideal assessment of immune function requires a set of tests that measure several different components. Studies have shown that the immune system of birds is sensitive to environmental contaminants that can cause immunotoxicity (Yunus *et al*., 2011). Aiming at better understanding mycotoxin immunotoxicity in birds, we evaluated the *in-vitro* cytotoxicity of a wide range of AFB_1 concentrations to peripheral lymphocytes of broiler chickens at different time points.

First, we investigated the role of AFB_1 on lymphocyte viability by measuring the dehydrogenase enzyme activity. MTT assay is dependent on respiratory chain activity (Mosmann, 1983), which is responsible for energy production in the form of ATP and it is necessary to maintain system organization and cellular functions. Our results with the MTT assay showed a strong correlation of mycotoxin concentration with the time of exposure. AFB_1 significantly reduced lymphocyte mitochondrial activity at 48 h and 72 h of incubation at the higher concentrations tested, possibly due to mitochondrial dysfunction (Bbosa *et al*., 2013). Consistent with this study, Taranu *et al*. (2010), in experiments with human and porcine lymphocytes, also found considerable reduction of lymphocyte cellular proliferation at doses 1 and 10 µg/mL of AFB_1. Contradictory results were reported by Bernabucci *et al*. (2011), who found increased viability of bovine lymphocytes in the presence of 20 µg/mL of AFB_1 on days 2 and 7 of incubation.

According to Fairbrother *et al*. (2004), reduced lymphoid cell count may suggest decreased immunological defense functions associated with those cells. On the other hand, circulating white blood cell counts may increase in response to infections, even in the presence of immunosupression. This possibility represents a limitation of the MTT assay, because when bioactive compounds, such as AFB_1, are analyzed, cellular metabolism can be altered. Moreover, the detection of cell-free DNA has emerged as an attractive tool in the early prognosis of several diseases. For this reason, we conducted the PicoGreen fluorescent assay to measure cell-free DNA circulating in cell supernatants, which would represent the extent of cell damage.

We observed a dose-dependent increase in dsDNA at 24 h of incubation. This result is consistent with the findings of Guengerich *et al*. (1998), who reported that highly reactive AFB_1-exo-8,9-epoxide is able to form adducts with DNA that are directly proportional to AFB_1 dose (Choy, 1993). DNA release may either stimulate or inhibit immune cell activation, depending on its concentration, sequence, and context (Scaffidi *et al*., 2002). Moreover, viable cells produce only limited amounts of extracellular DNA and necrotic cells produce little or none, while apoptotic cell death can be a significant source of extracellular DNA (Choi *et al*., 2004). Considering cell culture as a closed system, we hypothesized that apoptotic cell death was the source of dsDNA at 24 h, whereas immunostimulation can be related to the

interference with regulatory mechanisms that induce direct and indirect consequences on proliferation and differentiation of lymphocytes, as observed at 24 and 48 h (MTT assay). Our DNA damage findings agree with the results of Awad *et al*. (2012), who demonstrated that diets contaminated with the mycotoxin deoxynivalenol at moderate levels, in combination with low-protein feed, are able to induce lymphocyte DNA damage in chickens. Frankic *et al*. (2006) also demonstrated DNA fragmentation in chicken splenocytes treated with T-2 toxin and deoxynivalenol.

In the present study, ROS levels increased in AFB_1-treated lymphocytes of broiler chickens at 24 h of exposure at all the evaluated concentrations. It was shown that mycotoxin interaction in the oxidative stress induction is an early effect, which might be related to AFB_1 immunotoxicity. AFB_1 requires metabolic activation by CYP-450 to exert its cytotoxic and carcinogenic effects (Bbosa *et al*., 2013). CYP-450 activity itself is associated with electron leakage, and it is also one of the important sources of intracellular ROS generation (Shen *et al*., 1996). Therefore, when AFB_1 is bioactivated by this enzymatic complex, such process is expected to increase ROS production. Bischoff & Ramaiah (2007) described that the reactive species generated result in lipid peroxidation of membranes and oxidative modification of proteins and DNA in lesions. Increased ROS levels were also reported by Kouadio *et al*. (2005) in the human intestinal cell line Caco-2 following exposure to the mycotoxins fumonisin B_1 (FB_1), deoxynevalenol and zearalenone, and in rat spleen mononuclear cells treated with AFB_1 and FB_1.

In conclusion, the results of this study showed that the AFB_1 effects on broiler chicken immune system can be cytotoxic, depending on exposure time and dose. In addition, it was demonstrated that AFB_1 is able to affect the oxidative status of broiler chicken lymphocytes by increasing ROS levels, and to cause biomolecular oxidative damage, as evidenced by the PicoGreen assay. This study contributes to the use of the immune function as a biological marker of contamination effects in avian species, because immune cells such as lymphocytes can be important mediators of immunotoxic responses.

CONFLICTS OF INTEREST STATEMENT

There are no actual or potential conflicts of interest.

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