In-Vitro Cytotoxicity of Aflatoxin B₁ to Broiler Lymphocytes of Broiler Chickens

ABSTRACT

The aim of the present work was to study the in-vitro cytotoxic effects of different concentrations of aflatoxin B₁ (AFB₁) on broiler lymphocytes. Lymphocyte-rich mononuclear cells were separated by Ficoll-Histopaque density and cultured in 96-wellplates containing the evaluated AFB₁ concentrations in 5% CO₂ atmosphere at 39°C. Thereafter, MTT, PicoGreen, and reactive oxygen species assays were performed. Cell viability decreased in the presence of 10 µg/mL AFB₁ at 48 h (p < 0.05) and of 10 and 20 µg/mL AFB₁ 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 AFB₁ 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 B₁, B₂, G₁ and G₂ that are naturally present in many food products, and M₁ and M₂ are found in milk, dairy products, eggs, meat, and urine (Oliveira et al., 2000).

The toxigenic potential of aflatoxins depends both 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). AFB₁ 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 AFB₁ is responsible for the induction and modulation of diseases in humans and animals (Lewis et al., 1999). AFB₁ mainly affects cell-mediated immunity (Williams et al., 2004), reducing lymphocyte proliferation and cytokine production in experimental animals (Abbès et al., 2010). Moreover, AFB₁ 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 AFB₁ requires its metabolic activation by cytochrome (CYP) P-450, which is primarily responsible for the activation of AFB₁, to produce the ultimate carcinogen AFB₁-8,9-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 AFB1 exposure causes significant losses in the poultry industry (Sur & Celik, 2003). Contamination with AFB1 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 AFB1 on lymphoid cells of broiler chickens cultivated in vitro.

Materials and Methods

Chemicals

AFB1 (C17H12O6 - 5mg), RPMI-1640 medium, Ficoll-Histopaque density® (Sigma-Aldrich Co.), 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).

AFB1

AFB1 (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. AFB1 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 AFB1 treated samples, including the addition of the vehicle (0.5% ethanol) instead of AFB1.

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% CO2 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 105 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% CO2 at 39°C, and treated with increasing concentrations of AFB1 (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 AFB1 exposure. All tests were performed in 96-well microplates in triplicate. The results were expressed as optical density (OD540).

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 iTM, 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.

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<th>Time</th>
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<td>0 h</td>
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<tr>
<td>24 h</td>
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<tr>
<td>48 h</td>
<td>0.761 ± 0.02</td>
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<td>72 h</td>
<td>0.760 ± 0.01</td>
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<th>Time effect</th>
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* p < 0.05, ** p < 0.01, *** p < 0.001, compared to control group (0 μg/ml AFB1) within the time of exposure.

**RESULTS**

**MTT assay**

The lymphocyte viability of broiler chickens was assessed in the presence of AFB1, 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 AFB1 on free dsDNA in lymphocytes of broiler chickens were evaluated using the PicoGreen...
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First, we investigated the role of AFB1 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. AFB1 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 AFB1. Contradictory results were reported by Bernabucci et al. (2011), who found increased viability of bovine lymphocytes in the presence of 20 μg/mL of AFB1, 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 AFB1, 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 AFB1-exo-8,9-epoxide is able to form adducts with DNA that are directly proportional to AFB1 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 AFB1-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 AFB1 immunotoxicity. AFB1 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 AFB1 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 B1, (FB1), deoxynivalenol and zearalenone, and in rat spleen mononuclear cells treated with AFB1, and FB1.

In conclusion, the results of this study showed that the AFB1 effects on broiler chicken immune system can be cytotoxic, depending on exposure time and dose. In addition, it was demonstrated that AFB1 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.

ACKNOWLEDGEMENTS

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