



■ Author(s)

Zimmermann CEP^I
Machado AK^{II}
Cadoná FC^{II}
Jaques JAS^{II}
Schlemmer KB^I
Lautert C^{III}
Cruz IBM^{I,II}
Zanette RA^I
Leal DBR^{II}
Santurio JM^I

^I Programa de Pós-graduação em Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, Brazil.

^{II} Programa de Pós-graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, Brazil.

^{III} Faculdade de Veterinária/Setor de Micologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

■ Mail Address

Corresponding author e-mail address:
Prof. Dr. Janio Morais Santurio
Laboratório de Pesquisas Micológicas,
Departamento de Microbiologia e
Parasitologia, Campus UFSM, Prédio 20,
Sala 4139, Universidade Federal de Santa
Maria, 97105-900 Santa Maria, RS, Brazil.
Phone/Fax: + 55 55 3220 8906
E-mail: janio.santurio@gmail.com

■ Keywords

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

Submitted: October/2013

Approved: May/2014

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 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®, 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 (Mehrzaad *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 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 AFB₁) 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).

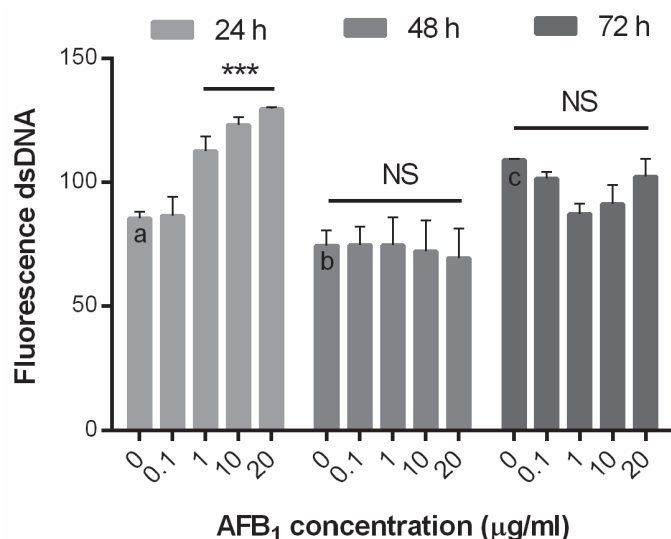


Figure 1. Free dsDNA quantification. Lymphocytes of broiler chickens were exposed to increasing concentrations of AFB₁ at 24, 48 and 72 h and dsDNA levels were measured by the PicoGreen assay. Data are means ± standard deviations. *** $p < 0.001$ compared to the control group (0 µg/ml AFB₁) at each time 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 (H₂O₂). 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 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



fluorescent assay (Fig. 1). An increase in dsDNA was observed at the AFB₁ 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 AFB₁ 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.

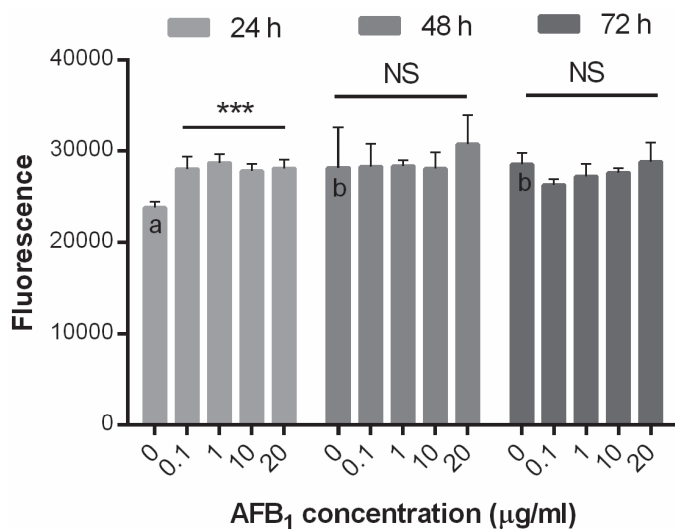


Figure 2. Intracellular formation of ROS measured by the DCFH-DA assay. Lymphocytes of broiler chicken were exposed to increasing concentrations of AFB₁ and the fluorescence intensity was monitored at 24, 48 and 72 h of incubation. Data are means \pm standard deviations. *** $p < 0.001$ compared to the 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 AFB₁ concentrations to peripheral lymphocytes of broiler chickens at different time points.

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 immunosuppression. 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), deoxynevalenol 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

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. R. A. Z. acknowledges FAPERGS for the grant.

REFERENCES

- Abbès S, Ben Salah-Abbès J, Abdel-Wahhab MA, Ouslati R. Immunotoxicological and biochemical effects of aflatoxins in rats prevented by Tunisian montmorillonite with reference to HSCAS. *Immunopharmacology and Immunotoxicology* 2010;32(3):514-522.
- Awad WA, Ghareeb K, Dadak L, Gille L, Staniek K, Hess M, Böhm J. Genotoxic effects of deoxynivalenol in broiler chickens fed low-protein feeds. *Poultry Science* 2012;91(3):550-5.
- Azzam AH, Gabal MA. Aflatoxin and immunity in layer hens. *Avian Pathology* 1998; 27(6):570-7.
- Bbosa GS, Kitya D, Lubega A, Ogwal-Okeng J, Anokbonggo WW, Kyegombe DB. Review of the biological and health effects of aflatoxins on body organs and body systems. In: Mehdi Razzaghi-Abyaneh M. *Aflatoxins - recent advances and future prospects*. InTech; 2013. chap. 12.
- Bernabucci U, Colavecchia L, Danieli PP, Basirico L, Lacetera N, Nardone A, Ronchi B. Aflatoxin B₁ and fumonisin B₁ affect the oxidative status of bovine peripheral blood mononuclear cells. *Toxicology in vitro* 2011;25(3):684-691.
- Bischoff K, Ramaiah K. Liver toxicity. In: Gupta RC, editor. *Veterinary toxicology basic and clinical principles*. New York: Elsevier Academic Press; 2007. p. 145-160.
- Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scandinavian Journal of Clinical and Laboratory Investigation* 1968; 97:77-89.
- Brasil. Ministério da Agricultura do Abastecimento e da Reforma Agrária; 2000. Instrução Normativa nº 3 de 17 de janeiro de 2000. Aprovar o Regulamento Técnico de Métodos de Insensibilização para o Abate Humanitário de Animais de Açougue. Brasília, DF; 2000. (corrigir no texto de Mapa 2000 para Brasil 2000)
- Choi JJ, Reich III CF, Pisetsky DS. Release of DNA from dead and dying lymphocyte and monocyte cell lines in vitro. *Scandinavian Journal of Immunology* 2004;60(1-2):159-166.
- Choy WN. A review of the dose-response induction of DNA adducts by aflatoxin B₁ and its implications to quantitative cancer-risk assessment. *Mutation Research* 1993;296(3):181-198.
- Fairbrother A, Smits J, Grasman KA. Avian immunotoxicology. *Journal of Toxicology and Environmental Health Part B: Critical Reviews* 2004;7(2):105-37.
- Frankic T, Pajk T, Rezar V, Levart A, Salobir J. The role of dietary nucleotides in reduction of DNA damage induced by T-2 toxin and deoxynivalenol in chicken leukocytes. *Food and Chemical Toxicology* 2006;44(11):1838-44.
- Gabal MA, Azzam AH. Interaction of aflatoxin in the feed and immunization against selected infectious diseases in poultry. II. Effect on one-day-old layer chicks simultaneously vaccinated against Newcastle disease,



- infectious bronchitis and infectious bursal disease. *Avian Pathology* 1998;27(3):290-295.
- Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouët S. Activation and detoxication of aflatoxin B₁. *Mutation Research* 1998;402(1-2):121-8.
- Halliwel B, Whiteman M. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *British Journal of Pharmacology* 2004;142(2):231-255.
- Hamid AS, Tesfamariam IG, Zhang Y, Zhang ZG. Aflatoxin B₁-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention. *Oncology Letters* 2013;5(4):1087-1092.
- Kalpana S, Aggarwal M, Srinivasa Rao G, Malik JK. Effects of aflatoxin B₁ on tissue residues of enrofloxacin and its metabolite ciprofloxacin in broiler chickens. *Environmental Toxicology and Pharmacology* 2012;33(2):121-6.
- Kouadio JH, Théophile AT, Baudrimont I, Moukha S, Dano SD, Creppy EE. Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B₁ in human intestinal cell line Caco-2. *Toxicology* 2005;213(1-2):56-65.
- LeBel C, Ischiropoulos H, Bondy S. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chemical Research in Toxicology* 1992;5(2):227-231.
- Lewis C, Smith J, Anderson J, Freshney R. Increased cytotoxicity of food-borne mycotoxins toward human cell lines *in vitro* via enhanced cytochrome p450 expression using the MTT bioassay. *Mycopathologia* 1999;148(2):97-102.
- Marai IFM, Asker AA. Aflatoxins in rabbit production: hazards and control. *Tropical and Subtropical Agroecosystems* 2008;8(1):1-28.
- Mehrzed J, Klein G, Kamphuesed J, Wolf P, Grabowski N, Schuberth HJ. *In vitro* effects of very low levels of aflatoxin B₁ on free radicals production and bactericidal activity of bovine blood neutrophils. *Veterinary Immunology and Immunopathology* 2011;141:16-25.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 1983;65(1-2):55-63.
- Nathanson RM. Transformation of chicken lymphocytes stimulated by concanavalin A: response parameters. *Canadian Journal of Comparative Medicine* 1982;46(1):57-59.
- Oliveira CAF, Kobashigawa E, Reis TA, Mestieri L, Albuquerque R, Corrêa B. Aflatoxin B₁ residues in eggs of laying hens fed a diet containing different levels of the mycotoxin. *Food Additives & Contaminants: Part A* 2000;17(6):459-462.
- Santurio JM. Mycotoxins and mycotoxicosis in poultry. *Revista Brasileira de Ciência Avícola* 2000;2(1):01-12.
- Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002;418(6894):191-5.
- Shen H, Shi C, Shen YI, Ong C. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B₁. *Free Radical Biology & Medicine* 1996;21(2):139-146.
- Sur E, Celik I. Effects of aflatoxin B₁ on the development of the bursa of Fabricius and blood lymphocyte acid phosphatase of the chicken. *British Poultry Science* 2003;44(4):558-566.
- Swarup V, Srivastava AK, Padma MV, Rajeswari MR. Quantification of circulating plasma DNA in Friedreich's ataxia and spinocerebellar ataxia types 2 and 12. *DNA Cell Biology* 2011; 30(6):389-394.
- Taranu I, Marina D, Burlacu R, Pinton P, Damian V, Oswald IP. Comparative aspects of *in vitro* proliferation of human and porcine lymphocytes exposed to mycotoxins. *Archives of Animal Nutrition* 2010;64(5):383-393.
- Williams J, Phillips T, Jolly P, Stiles J, Jolly C, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American Journal of Clinical Nutrition* 2004;80(5):1106-22.
- Yunus AW, Razzazi-Fazeli E, Bohm J. Aflatoxin B₁ in affecting broiler's performance, immunity, and gastrointestinal tract: a review of history and contemporary issues. *Poultry Science* 2011;90:1683-89.