Toxicology in Vitro 29 (2015) 538–543

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Cytoprotective and genoprotective effects of β-glucans against aflatoxin B_1-induced DNA damage in broiler chicken lymphocytes

C.E.P. Zimmermann [a], I.B.M. Cruz [a,b], F.C. Cadoná [b], A.K. Machado [a], C. Assmann [c], K.B. Schlemmer [a], R.A. Zanette [a], D.B.R. Leal [b], J.M. Santurio [a,*]

[a] Programa de Pós-Graduação em Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Laboratório de Biogenômica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 28 June 2014
Accepted 9 January 2015
Available online 20 January 2015

Keywords:
β-glucans
Aflatoxin B_1
Cytotoxicity
Genotoxicity
Oxidative stress

ABSTRACT

The polysaccharide β-glucan presents beneficial effects on the immune system, although the mechanisms of the immunomodulatory effect remain poorly understood. The potential cytoprotective and genoprotective effects of β-glucans were evaluated in broiler chicken lymphocytes exposed to increasing concentrations of aflatoxin B_1 (AFB_1) and/or β-glucans. AFB_1 significantly decreased cell viability at the concentrations of 10 and 20 μg/ml at 72 h of incubation ($p < 0.01$ and $p < 0.001$, respectively). Moreover, the AFB_1 concentrations of 1, 10 and 20 μg/ml increased DNA fragmentation levels at 24 h ($p < 0.001$). Conversely, lymphocyte death was prevented by β-glucans at the concentrations of 1% and 10%, indicating a cytoprotective effect. Reactive oxygen species levels were increased in the cells treated with 20 μg/ml AFB_1 at 24 h ($p < 0.05$) and 10% β-glucans with or without AFB_1 at 24, 48 and 72 h of incubation ($p < 0.001$). DNA damage increased by more than 100% in AFB_1-treated lymphocytes when compared to control group. β-glucans at 1% was able to fully revert the AFB_1-induced lymphocyte DNA damage, indicating a genoprotective effect and maintaining DNA integrity. In conclusion, β-glucans showed *in vitro* dose-dependent cytoprotective and genoprotective effects in broiler chicken lymphocytes exposed to AFB_1.

1. Introduction

Mycotoxins such as aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* are commonly found in grains and other foods and feedstuffs, causing health impairment and economic losses (Iheshiulor et al., 2011). AFB_1 is responsible for losses in animal productivity, especially in broiler chickens (Hamid et al., 2013; Zimmermann et al., 2014), causing fatty liver and kidney disorders, leg and bone problems, alterations in pigmentation (carcasses, egg yolk), reduced hatchability, smaller eggs and immunity dysfunctions that lead to vaccine failure and lower resistance to diseases (Iheshiulor et al., 2011).

Chronic exposure to AFB_1 is highly mutagenic and associated with human hepatocellular carcinoma (Hamid et al., 2013). The risk is related to AFB_1 metabolization by cytochrome (CYP) P-450 enzymes that generates toxic metabolites such as AFB_1-8,9-exo-epoxide (Marin and Taranu, 2012), a genotoxic hepatocarcinogen that presumptively causes cancer by inducing DNA adducts, leading to genetic changes in the target cells and oxidative damage (Mary et al., 2012; Verma, 2004).

Growth-promoting antibiotics, commonly used in poultry production, have been replaced by prebiotic or probiotic agents because of the European ban on the use of antibiotics in animal feed. Therefore, there has been an urge to the development of alternative methods to promote animal health (Phillips, 2007; Rieder et al., 2013). Studies have demonstrated that dietary immunomodulators such as β-glucans show beneficial results in a wide variety of animal species (Chae et al., 2006; Guo et al., 2003). The immunostimulant and immunomodulatory effects of β-glucan polysaccharides result in the regeneration of the host's ability to resist life-threatening opportunistic infections (Rieder et al., 2013). The immunomodulatory process initiates when the β-glucan binds to

Abbreviations: AFB_1, aflatoxin B_1; DCFH-DA, 2′,7′ dichlorofluorescein diacetate; dsDNA, double-stranded DNA; ROS, reactive oxygen species.

* Corresponding author at: 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. Tel./fax: +55 55 3220 8906.

E-mail address: janio.santurio@gmail.com (J.M. Santurio).

http://dx.doi.org/10.1016/j.tiv.2015.01.005
0887-2333/

cell surface receptors of macrophages, lymphocytes and neutrophils (Chen and Seviour, 2007; Guo et al., 2003). β-glucan activates B-lymphocytes and macrophages through dectin-1, CR3, lactosylceramide, scavenger receptors and Toll-like receptors (Le et al., 2011; Taylor et al., 2002), modulating the immune system and inducing the production of cytokines (Cheng et al., 2004). In broilers, β-glucan has been shown to be an excellent adjuvant for the avian influenza H5 subtype vaccine, enhancing the vaccine immunogenicity (Le et al., 2011).

Recently, Zimmermann et al. (2014) showed that AFB_1 can be cytotoxic and to cause biomolecular oxidative damage in broiler chicken lymphocytes. Therefore, the present study was designed to examine the *in vitro* cytoprotective and genoprotective effects of β-glucans (term β-glucan will be used throughout the paper) on broiler chicken lymphocytes exposed to different concentrations of AFB_1.

2. Materials and methods

2.1. Chemicals

AFB_1 ($C_{17}H_{12}O_6$), RPMI 1640 medium, Ficoll-Histopaque™, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate, penicillin/streptomycin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), 2′,7′ dichlorofluorescein diacetate (DCFH-DA) and β-glucan (25 mg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum was obtained from Cultilab (Campinas, SP, BR) and Quant-iT™ PicoGreen® reagent from Invitrogen (Eugene, UK).

2.2. Broiler chicken lymphocyte culture

Lymphocytes were obtained from adult broiler chickens (38–42 days, 2–2.5 kg) from a local slaughterhouse. All experimental procedures were conducted according to Normative Instruction N° 3, January 17th, 2000 (Regulation of Technical Methods for the Humane Slaughter of Animals) (MAPA, 2000). The animals were submitted to electronarcosis, followed by a bleeding operation that was performed by sectioning the large vessels of the neck. The blood was collected in sterile culture tubes containing dipotassium EDTA as anticoagulant.

Broiler chicken lymphocyte culture was performed as described by Nathanson (1982), with some modifications. Blood mononuclear cells were isolated from samples using Ficoll-Histopaque density. These cells were centrifuged at 1000 rpm for 5 min with culture medium to completely remove the 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 occur adhesion of monocytes, which can eventually be among the non-adherent lymphocytes, to the surface of the bottle. After incubation, cell suspensions were transferred into centrifuge tubes and centrifuged at 1500 rpm for 10 min. The pellets containing lymphocytes were suspended in 3 ml of culture medium, and the cell viability was measured using trypan blue dye (1:2). Lymphocytes were suspended at a density of 0.7×10^5 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.

2.3. AFB_1 and β-glucan treatment conditions

AFB_1 (5 mg) was dissolved in 99% ethanol and β-glucan (25 mg) was dissolved in saline 0.9% and subjected to sonic energy via a 19-mm probe using a 300-V/T Sonic Dismembrator for 30 min, alternating with water bath at 80 °C for 10 min (Gonzaga et al., 2009; Mehrzad et al., 2011). Further dilutions were made in RPMI 1640 medium containing 9.7 mM HEPES and 24 mM sodium bicarbonate and 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. β-glucan was added to the medium at the concentrations of 0.1, 1 and 10% v/v. The lymphocyte samples were exposed to the concentrations of AFB_1 and/or of β-glucan. The density of the cells that were seeded was equivalent to 75% confluence. Control group cells were prepared in the same manner as treated samples, including the addition of the vehicle (0.5% ethanol) but in the absence of AFB_1 and β-glucan. The potential beneficial effects of β-glucan on broiler chicken lymphocytes were evaluated by the MTT, PicoGreen, DCFH-DA and comet assays using the highest AFB_1 concentration (20 μg/ml).

2.4. MTT viability assay

The MTT bioassay was performed to monitor cell viability and to analyze the functional and proliferative characteristics of lymphocytes. The MTT assay 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 at 540 nm was measured by a microplate spectrophotometer. The cytotoxic effect of AFB_1 was evaluated after 24, 48 and 72 h of incubation, and the protective effect of the β-glucan concentrations of 0.1%, 1% and 10% was evaluated in lymphocytes exposed to the highest AFB_1 concentration (20 μg/ml) at 72 h. This assay was performed in 96-well microplates, in triplicate. The results were expressed as the percentage of the absorbance values of the control group (0 μg/ml AFB_1 and 0% β-glucan) to the absorbance of the treated groups.

2.5. Double-stranded DNA (dsDNA) levels cytotoxicity assay

The PicoGreen fluorescence assay measures the presence of dsDNA fragmentation, which is also an indicative of cytotoxicity (Swarup et al., 2011). This assay was performed only after 24 h of exposure and followed the protocol supplied by the manufacturer. PicoGreen dye was diluted to 1:200 with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and incubated with each supernatant sample in black 96-well microplates in the dark at room temperature 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, the average of which was subtracted from the average fluorescence of the other samples. The results were expressed as % of control fluorescence using the following equation:

$$\%\ \text{of control} = [(\text{sample fluorescence} \times 100)/\text{mean of control sample fluorescence}].$$

2.6. Determination of intracellular reactive oxygen species (ROS) using DCFH-DA

The effects of AFB_1 exposition with and without β-glucan supplementation on ROS levels of broiler chicken lymphocyte cultures were determined using the non-fluorescent cell permeating