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Effect of partially purified fumonisins on cellular immune response in experimental murine paracoccidioidomycosis

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Fumonisins are mycotoxins produced mainly by *Fusarium verticillioides*, which can modulate the immune response. Paracoccidioidomycosis (PCM), caused by the fungus *Paracoccodioides brasiliensis* (Pb), is one of the most important systemic mycoses in Latin America. The aim of this study was to evaluate the effect of the partially purified fumonisins on cellular immune response in mice infected with Pb. Four groups of male BALB/c mice were used. Groups PB and PB/FB were inoculated i.v. with 1×10^5 Pb yeast cells and, after 28 days, groups FB and PB/FB were inoculated (s.c.) with partially purified fumonisin B_1 from *F. verticillioides* (5 × 2.25 mg FB1/kg body weight). After 7 days, cellular immune response was evaluated by delayed-type hypersensitivity (DTH) and lymphoproliferative assays (LA) using spleen cells. Nitric oxide (NO) production by spleen cells was also evaluated. The specific LA response to Pb antigen was higher in group PB than in FB and CTR groups ($p < 0.05$) but not significant with PB/FB. The DTH response was higher in infected than non infected groups ($p<0.05$) but also no significantly with PB and PB/FB groups. The lyphoproliferative response to ConA was decreased in FB or PB/FB in relation to CTR ($p<0.05$) but not with PB/FB and also a reduction of NO levels was observed in fumonisin treated in relation to control group FB1/kg ($p<0.05$). In conclusion, fumonisin B_1 or other components of *F. verticillioides* extracts significantly suppress the unspecific cellular immune response and the NO production by splenocytes from *P. brasiliensis* infected or not infected BALB/c mice.

Key words: Fumonisin, *Paracoccodioides brasiliensis*, lymphoproliferative assay, nitric oxide.

INTRODUCTION

Fumonisins are a group of mycotoxins produced by the plant pathogen *Fusarium verticillioides* and found predominantly in maize (Shephard et al., 1996). The extent of contamination of raw corn with fumonisins varies with geographic location, agricultural and storage practices and the vulnerability of the plants to fungal invasion

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during all phases of growth, storage and processing (Shelby et al., 1994). Brazil produces and consumes very large quantities of maize in a variety of forms, and Ono et al. (2000, 2001) reported fumonisin contamination in 98% of analyzed samples. Paracoccidioidomycosis (PCM) is a granulomatous disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Pb). The mycosis is endemic in Latin America and the majority of cases are reported in Brazil, where it is the 8th commonest cause of mortality among infectious diseases (Coutinho et al., 2002). PCM affects preferentially male farm workers (Coutinho et al., 2002; Franco et al.,1989). Airborne fungal propagules, consisting of conidia or hyphal fragments, begin the infection and undergo con-version to the yeast phase, the infective stage of PCM, in the lungs, progressing to haematogenic or lymphatic dissemination to the liver, spleen, skin and mucosa (McEwen et al., 1987). The disease may be developed in multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal), depending on the host immune response (Marques et al., 2002). Fumonisin B_1 (FB_1) and their analogs are secondary toxic metabolic produced, mainly by *F. verticillioides* and *F. proliferatum* in maize (Leslie, 1996). The ingestion of FB_1 is related to many pathologies in humans as well as mice, rats, equines and swines (Sydenham et al., 1990; Ueno et al., 1997; Howard et al., 2001; Gelderblom et al., 1991; Marasas et al., 1988; Colvin and Harrison, 1992; Vizcarra-Olvera et al., 2012, Khan et al., 2012). The FB_1 can modulate the cellular or humoral immune response, affecting the antigen presenting cells, B and T lymphocytes and macrophages (Liu et al., 2002; Taranu et al., 2006; Martinova and Merrill, 1995; Devriendt, et al., 2009, Grenier et al., 2011, Bracarense et al., 2012, Burel et al., 2013). Fumonisin B_1 (FB_1) and their analogs are secon-dary toxic metabolic produced, mainly by *F. verticillioides* and *F. proliferatum* in maize (Leslie, 1996). The ingestion of FB_1 is related to much pathology in humans as well as mice, rats, equines and swines (Sydenham et al., 1990; Ueno et al., 1997; Howard et al., 2001; Gelderblom et al., 1991; Marasas et al., 1988; Colvin and Harrison, 1992). The FB_1 can modulate the cellular or humoral immune response, affecting the B and T lymphocytes activities and can also disrupt effector function of macrophages (Liu et al., 2002; Taranu et al., 2006; Martinova and Merrill, 1995). Due to chronicicity of PCM and its preva-lence in Brazil, there is a possibility of the concomitant contamination of fumonisin and Pb, mainly, maize is used as a basic feeding for the local poor community who are involved greatly in agriculture and the illness also affects the agricultural and poor population. In a previus experi-mental study, it was observed significantly increased the specific antibody response in male Swiss mice infected with Pb by FB_1 or other components of *F. verticillioides* extracts (Itano et al., 2008).

Considering the cellular immune response as the main defense mechanism against Pb (Silva et al., 1995). The aim of this study was to evaluate the effect of the partially purified fumonisins on cellular immune response in mice infected with Pb-infected BALB/c mice.

MATERIALS AND METHODS

Fumonisin production and determination

F. verticillioides 103F (isolated from feed involved in animal intoxication in 1991 at Paraná State, Brazil) was grown on potato dextrose agar (PDA) slants at 25°C for 7 days. An aliquot of conidia suspension (10^6 conidia mL^{-1}) was inoculated into three 500 ml Erlenmeyer flasks containing 100 g ground maize adjusted to 42% moisture content and autoclaved twice for 30 min. The culture was incubated at 25°C for 14 days, extracted twice by shaking with ethyl acetate (1 L/kg) and filtered. Next, the residue was extracted once with 1 L/kg methanol: water (3:1, v/v) and twice with 1.5 L/kg of the same solvent. The dried extract was dissolved in 200 ml methanol: water (1:3, v/v) at 50°C and partitioned three times with 100 ml chloroform. The aqueous phase was dried at 50°C under vacuum (Cawood et al., 1991). This residue was dissolved in 20 ml methanol: water (1:3, v/v), and partially purified by passing successively through columns of Amberlite XAD-2 (100 g, 2.0 × 60 cm, Supelco, 10357-EA), silica gel (120 g, 2.0 × 60 cm, Sigma, 70 to 230 mesh, S2509), silica gel (120 g, 2.0 × 60 cm, Sigma, 70 to 230 mesh, S2509) and reversed-phase C18 (50 g, 3.0 × 30 cm, Acros, S2425) columns, using methanol, chloroform: methanol: acetic acid (6:3:1, v/v/v), chloroform: methanol: water: acetic acid (55:36:8:1, v/v/v/v) and a linear gradient from methanol: water (1:1, v/v) to methanol: water (4:1, v/v) as the mobile phases, respectively.

Fumonisins were determined by high-performance liquid chromatography (HPLC) (Shephard et al., 1990) with some modifications (Ueno et al., 1993). An aliquot of partially purified extract (6 ml) was filtered through a 0.45-μm syringe filter (Millipore, Windham, US) and suitably diluted with acetonitrile: water (1:1, v/v). After derivatization with 200-μL of *o*-phthaldialdehyde (OPA) reagent, the sample was injected into the HPLC system within 1 min. Fumonisins were analyzed by a reversed-phase, isocratic system (Shimadzu LC-10 AD pump and RF-10A XL fluorescence detector), using a Shim-pack CLC-ODS (M) column (4.6 × 250 mm, Shimadzu). Fluorescence excitation and emission wavelengths were 335 and 450 nm, respectively. The eluent was CH_3OH: 0.1 M NaH_2PO_4 (80:20, v/v), adjusted to pH 3.3 with H_3PO_4 solution, flowing at 1 ml/min. The detection limits for the fumonisins FB_1 and FB_2 were 27.5 and 35.3 ng/g, respectively. FB_1 and FB_2 levels in the partially purified extract were 152.1 and 53.34 mg/mL, respectively. The sample was diluted in sterile PBS to a concentration of 22.5 mg FB_1/ml, this partially purified fumonisin B_1 (PPF) was used for animal treating.

P. brasiliensis

P. brasiliensis strain 18 (Pb 18) was cultured on Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil), and maintained at 35°C, subculturing at 5 day intervals. The yeast cells were collected in sterile saline, filtered through cotton and gauze layers, and the concentration was adjusted to 1×10^6 cells/ml.

Experimental design

Male BALB/c mice (n = 24), 4 to 5 weeks old (20 to 25 g), provided by the animal facilities of Londrina State University (UEL), were kept in a 12:12 h light-dark cycle at 25°C, with free access to sterilized commercial mouse food (Nuvital, Curitiba, Brazil) and water. Four groups of 6 mice were used: infected (PB), treated (FB),

infected and treated (PB/FB) and uninfected and untreated as control (CTR). Groups PB and PB/FB were inoculated (i.v.) with 1 × 10^5 yeast cells (Pb 18) and, 28 days later, groups FB and PB/FB were inoculated (s.c.) with PPF from *F. verticillioides* (5 × 2.25 mg FB1/kg body weight/day), as described by Johnson and Sharma (2001). The control group was inoculated with sterile PBS. Plasma samples and spleens cells were taken 7 days after PPF inoculation. The procedures and experimental design were approved by the Animal Research Ethics Committee of the UEL.

Exoantigen (exoAg)

A lyophilized exoAg was prepared from a yeast-phase culture of Pb strain B-339 according to Camargo et al. (1989).

Lymphoproliferation assay

Spleen cells from infected and normal mice were removed aseptically and teased. The erythrocytes were lysed with tris-ammonium chloride, and the cell suspension was washed three times in RPMI medium. For lymphoproliferative assays, 100 µl of the cells were cultured in triplicate wells at a concentration of 1 × 10^6 cells/ml in RPMI 1640 containing L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA), 10% fetal calf serum (Gibco, USA) in 96-well flat-bottom culture plates. ExoAg or concanavalin A (Con) (Sigma-Aldrich, St. Louis, Missouri, USA) was then added to each well at concentrations of 50 and 10 µg/ml, respectively and as control with RPMI medium. The cells were cultured for 3 (ConA) or 5 (exoAg) days at 37°C with 5% CO_2 and were pulsed with 1 µCi of (^{3}H) thymidine 18 h before harvesting on glass filter strips. The radioactivity was determined by a liquid scintillation counter (Beckman LS 6.800). The 'stimulation index' was calculated as the triplicate of stimulated cells (cells + ExoAg or ConA) divided by the cell control (cell + RPMI).

Delayed-type hypersensitivity assay (DTH)

The DTH reactions were performed in all groups of animals. Mice were inoculated with exoAg (10 µg) and sterile PBS in right and left footpad respectively. The footpad thickness was measured at 24 h after inoculation with caliper (Mitotoyo, Tokyo, Japan). The increase in thickness was calculated, as in the formula ($A = D^2/4$, = 3.14).

Measurement of nitric oxide (NO)

The concentration of NO in spleens cells was measured by a microplate Griess assay. Spleen cells (100 µL) prepared as described earlier, were cultured with lipopolysaccharides (LPS), (7 µg) during 24 h at 37°C. The supernatants were collected and 50 µL of these samples were transferred to wells of 96-well flat bottom microtiter plate and incubated with an equal volume of the Griess reagent for 10 min at room temperature. The absorbance at 550 nm was determined with a Titertek Multiskan apparatus (Multiskan EX, Uniscience – Labsystems, Helsinki, Finland). The NO concentration was determined using a standard curve determined with different concentrations of $NaNO_2$.

Statistical analysis

The data were analysed by using a Tukey-Kramer ANOVA test for comparisons and declaring significance at $p<0.05$.

RESULTS

Proliferation response of spleens cells to exoAg and to ConA

During the present investigation, higher proliferation response to exoAg in infected groups (PB: 2.04 ± 0.71; PB/FB: 1.55 ± 0.26) in relation to not infected (FB: 0.87 ± 0.15; CTR; 1.10 ± 0.23) was observed (Figure 1). The decreased proliferation response in PB/FB was not statistically significant in relation to PB group. CTR × PB ($p<0.01$), FB × PB ($p<0.001$) and FB × PB/FB ($p<0.05$). Figure 2 shows decreased proliferation response to ConA in FB treated groups, both in infected or not infected (FB: 0.12 ± 0.08 and PB/FB: 0.17 ± 0.24) in relation to not treated (PB: 1.52 ± 0.28 and CTR:1.95 ± 0.41). CTR × FB and PB/FB, $p < 0.001$.

DTH assay

Results regarding the 'delayed type hypersensitivity' in the infected groups showed higher inflammatory response than control not infected group (PB: 1.59 ± 0.19; PB/FB: 1.49 ± 0.09, FB: 0.98 ± 0.08; CTR: 1.10 ± 0.16;), $p < 0.001$, but these results are similar between PB and PB/FB, $p > 0.05$ (Figure 3).

NO concentration in culture supernatants of spleens cells stimulated with LPS

During the present study, 'nitrous oxide' mount was measured in the supernatants of spleens stimulated with LPS and it was detected decreased level in FB treated or infected with Pb or infected and treated with PPF in relation to control group ($p < 0.001$), but similar between FB treated or infected with Pb or infected and treated with PPF ($p > 0.05$) (Figure 4).

DISCUSSION

In PCM, the cellular immune response represents the main defense mechanism against Pb and in this study, it was observed an induction of specific cellular response in infected mice both by DTH and LA assays. However, it was not observed significant supression in simultaneous infection with Pb and contaminated with PPF from *F. verticillioides* in experimental PCM in male BALB/c mice. However, there were a significant supression of response to ConA in mice treated with PPF in relation to the normal control without treatment with PPF, suggesting that the FB_1 in our conditions of experiment does not affect the specific cellular immune response but can suppress in specific immune response. Our results are partially in accordance with the data of Theumer et al. (2002), which

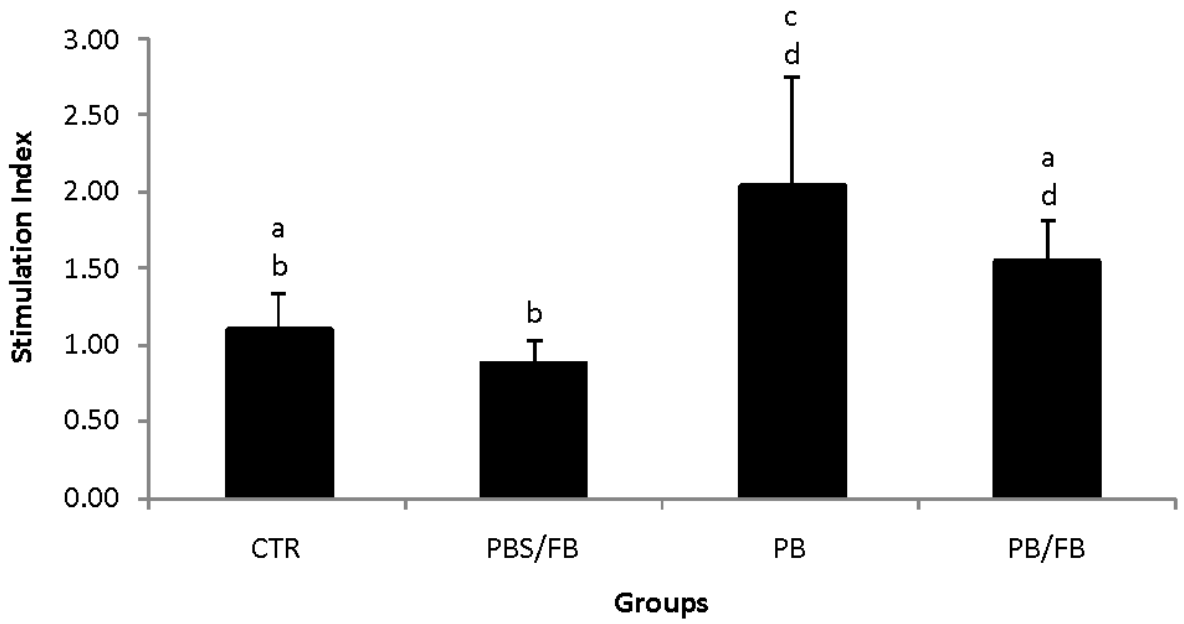

Figure 1. Proliferation response of spleens cells to exoAg. Spleen cells (1 × 105 cells/ml) were cultured with *P. brasiliensis* exoAg (50 µg/ml) for 5 days, pulsed with 1 µCi and the stimulation index calculated as the triplicate of stimulated cells divided by the control not stimulated cells. A higher proliferation response to exoAg was observed in infected groups in relation to not infected. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. Different letters indicate significant differences between groups ($p<0.05$).

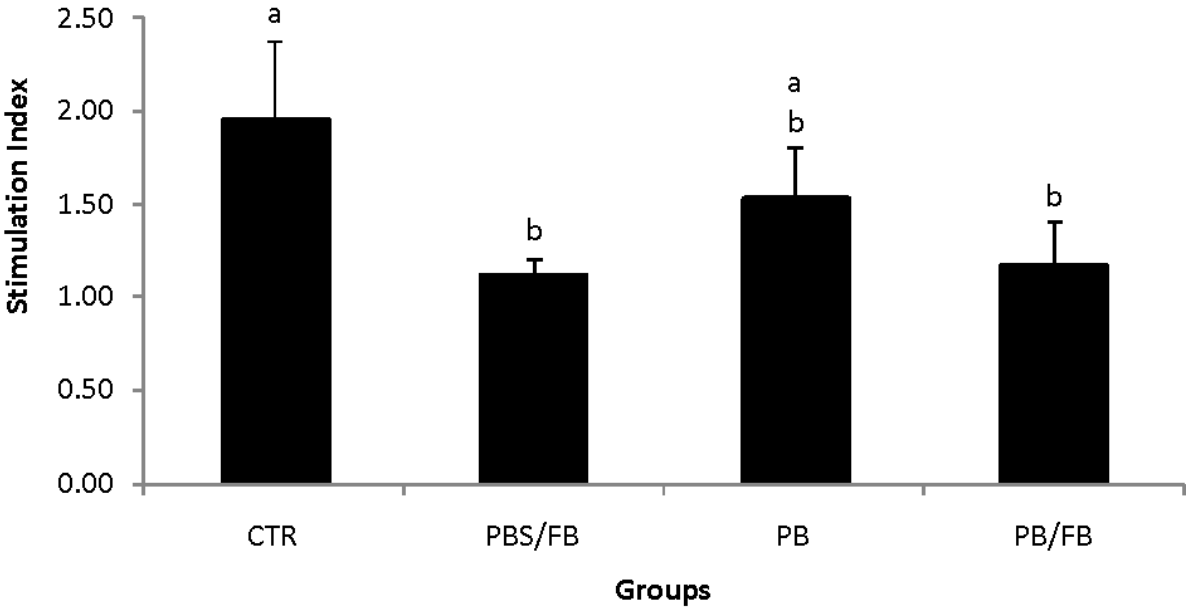

Figure 2. Stimulation of cellular proliferation with ConA. Spleen cells (1 × 10^5 cells) were cultured with ConA (10 µg/ml) for 72 h, pulsed with 1 µCi and the stimulation index (SI) calculated as the triplicate of stimulated cells divided by the control not stimulated cells. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 10^5 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB_1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. SI were more elevated in CTR and Pb groups than PBS/FB and PB/FB groups; however, significant results were found only between CTR and PBS/FB, and between PBS and Pb/FB. Different letters indicate significant differences between groups ($p<0.05$).

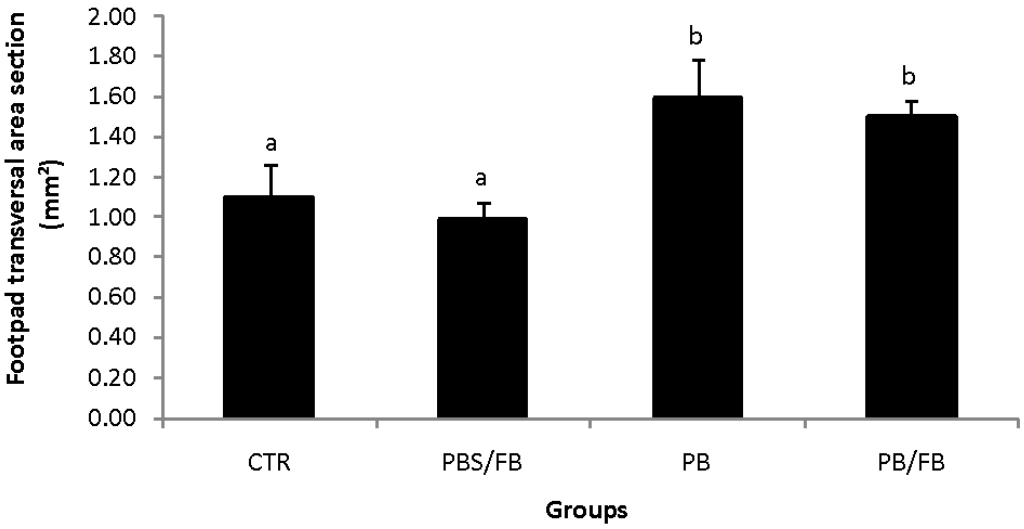

Figure 3. DTH assay. BALB/c mice were infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day) (PB/FB) or only infected (PB) or only treated with PPF (FB) or inoculated with sterile PBS (CTR). ExoAg from *P. brasiliensis* were injected intra-footpad 24 h before measurement of the footpad response. Different letters indicate significant differences between groups ($p<0.05$).

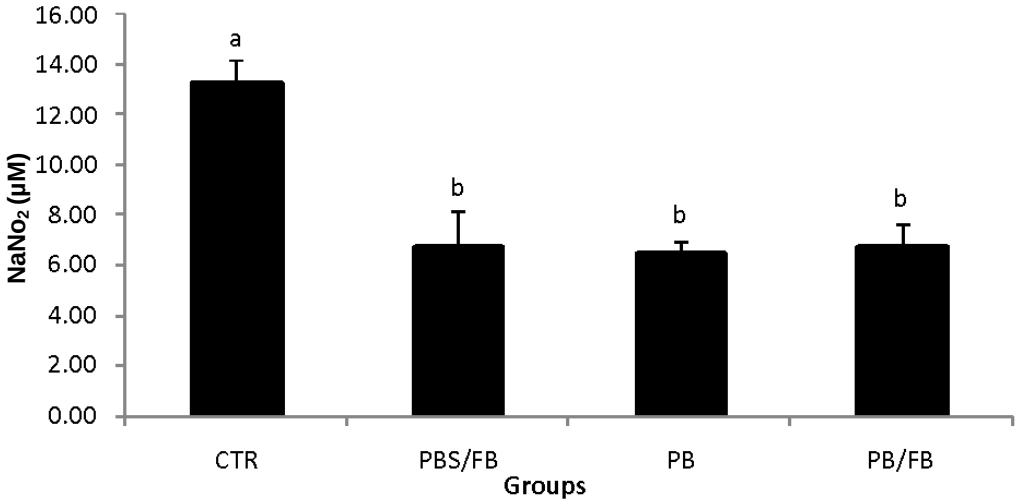

Figure 4. NO determination. Spleen cells were stimulated with LPS to determine the release of NO in the culture medium. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. Inhibition of NO was seen in groups infected and treated with PPF. Different letters indicate significant differences between groups ($p<0.05$).

they had observed no significant effect on mitogen-induced proliferation of spleen cells in mice treated with FB_1 and stimulated with phytohemaglutinin mitogen (PHA). The inflammatory reaction is a non-specific response and Gonzalez et al. (2000) reported that the NO produced by spleen macrophages has crucial role in *P. brasiliensis* defense. Our NO analysis demonstrated decreased levels when spleen macrophages from groups of infected or PPF treated or infected/treated mice stimulated with LPS in relation to control not treated and not infected group, suggesting that both PPF and Pb can modulate the NO production by spleen macrophages, but not with synergic

effect. Also, Popi et al. (2004) reported the decreased NO production by peritoneal macrophages incubated with gp43 and stimulated with zymozan.

The infection was confirmed by histologic analysis of lungs and liver, where no significant difference between the infected and infected/treated group was observed (data not shown), suggesting that in the conditions of the experiment the mycotoxin did not affect the gravity of the illness. All groups of animals had the same initial healthy appearance, which they maintained throughout the period of the study. This finding is in accordance with the results of evaluation of the specific cellular immune response, the main protective response against the Pb (Silva et al., 1995; Calich and Kashino, 1998; Kashino et al., 2000). In Bhandari et al. (2001) work, it was reported that FB_1 modulates the IFN-g, a Th1 marker cytokine in BALB/c mice according to the sex, with elevated expression (male) or down-modulation (female). The lack of change in specific cellular immune response in BALB/c mice (male) infected and treated with partially purified fumonisin could be due to the balance between FB_1 (Bhandari et al., 2001) and immunossupressive Pb antigens actions (Benard et al., 1997; Cacere et al., 2002 and Rigobello et al., 2013), that require further study.

The literature data has shown the low proliferation of spleenocytes to some Pb antigens (Benard et al., 1997), that induce apoptosis and sugested that it may be one of the mechanisms leading to hyporesponsiveness (Cacere et al., 2002). The relevance of this study stems from the fact that PCM is the 8th most common cause of death from predominantly chronic or recurring infectious and parasitic diseases in Brazil. It is believed that about 10 million people are infected with the fungus, and 2% of them may develop the disease (McEwen et al., 1995). Thus, any factor that leads to modulation of the immune response, such as the presence of mycotoxin, could contribute to the development of the disease, increase its severity or control the disease. For the best understanding about the action of the FB1 in the course of the experimental PCM, there are necessary additional studies using purified FB_1 in diverse concentrations/times of incubation/mouse isogenic lines by using both sex. We concluded for the results that FB or other components of *F. verticillioides* extracts does not affect specific cellular immune response in experimental PCM in male BALB/c mice, but can affect non-specific response, in the conditions of the work.

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