Influence of n-3 Polyunsaturated Fatty Acid in the Proliferative Activity of Lymphocytes During Experimental Infection with *Paracoccidioides brasiliensis*

Vinícius João Navarini¹, Sheisa Cyléia Sargi², Jesuí Vergílio Visentainer², Camila Freitas Oliveira¹ and Jeane Eliete Laguila Visentainer¹*

¹Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá, Av. Colombo, 5790, cep 87020-900, Maringá, Paraná, Brazil.
²Departamento de Ciências Agrárias - Ciência Gastronomica Universidade Estadual de Maringá, Maringá, Paraná, Brazil.*Author for correspondence. E-mail: jelvisentainer@gmail.com

ABSTRACT. It has been shown that diets containing polyunsaturated omega-3 fatty acids may modulate the immune system. Paracoccidioidomycosis (PCM) is a systemic mycosis of great importance in Latin America, caused by the thermo-dimorphic fungus *Paracoccidioides brasiliensis*. The aim of this study was to evaluate the influence of n-3 PUFAs in lymphoproliferative activity in murine experimental PCM. Spleen lymphocytes from animals infected with an isolate of *P. brasiliensis* (Pb18) and fed with diets supplemented with flaxseed oil or a commercial diet for eight weeks, were obtained and cultured for evaluation of lymphoproliferative activity by MTT assay. In uninfected animals and those fed an enriched-flaxseed diet the evaluated lymphoproliferative activity was inhibited in the first week; however, this activity resumed at later stages. On the other hand, in animals infected and fed an enriched-flaxseed diet, the interaction between the stimulus given by the presence of the fungus and the action of n-3 PUFAs appeared to stimulate lymphocyte proliferation after the first week of infection. n-3 PUFAs are shown to influence lymphoproliferative activity of mice in experimental PCM.

Keywords: omega-3, paracoccidioidomycosis, immune cell.

Influência do ácido graxo poli-insaturado n-3 na atividade proliferativa de linfócitos durante a infecção com *Paracoccidioides brasiliensis*

RESUMO. Tem-se demonstrado que dietas contendo ácido graxo poli-insaturado ômega-3 podem modular o sistema imune. A Paracoccidioidomicose é uma micose sistêmica de grande importância na América Latina, causada pelo fungo termo-dimórfico *Paracoccidioides brasiliensis*. O objetivo do estudo foi avaliar a influência do AGPI n-3 na atividade linfoproliferativa na PCM experimental murina. Linfócitos esplênicos de animais infectados com o isolado de *P. brasiliensis* e alimentados com uma dieta suplementada com óleo de linhaça por 8 semanas, foram obtidos e cultivados para avaliação da atividade linfoproliferativa através do ensaio de MTT. Nos animais não infectados e alimentados com a dieta enriquecida com linhaça houve a inibição da atividade linfoproliferativa na primeira semana, sendo retomada no estágio mais tardio. Por outro lado, nos animais infectados e suplementados com a dieta com óleo de linhaça, a interação entre o estímulo dado pela presença do fungo e a ação do AGPI n-3 parecem ter estimulado a proliferação de linfócitos depois da primeira semana de infecção. AGPI n-3 podem ter influenciado a atividade linfoproliferativa dos camundongos na PCM experimental.

Palavras-chave: ômega-3, paracoccidioidomicose, células imune.

Introduction

There is great interest in the study of polyunsaturated fatty acids (PUFAs), especially omega-6 (n-6) and omega-3 (n-3) PUFAs, whose precursors are linoleic acid (18:2 n-6; LA) and alpha-linolenic acid (18:3 n-3; LNA), respectively, due to the fact that they have beneficial effects on human health, especially on immune and inflammatory response (Perini et al., 2010; Hubbard, Chapkin, & Erickson, 1994). These fatty acids, through desaturation and elongation reactions, are converted to arachidonic acid (20:4 n-6; AA), eicosapentaenoic acid (20:5 n-3; EPA) and docosahexaenoic (22:6 n-3; DHA) acid (Chen, Wang, & Thompson, 2006).

These fatty acids are metabolized by the cyclooxygenase pathway which gives rise to prostaglandins (PG), thromboxanes (TX) and prostacyclin and lipoxigenase (LOX) pathway that synthesizes leukotrienes (LT). LA is metabolized to...
AA which is a precursor of inflammatory eicosanoids such as PGE₂, TXA₂ and LTB₄. PGE₂ has an immunosuppressive effect, due to the inhibition of lymphocyte proliferation and natural killer (NK) cells and production of interleukin 2 and interferon gamma (IFN-γ). PGE₂ is also able to inhibit the production of tumor necrosis factor alpha and members of the interleukin-1 family. Moreover, LNA is metabolized to EPA and DHA, which are precursors of inflammatory eicosanoids such as PGE₃ and PGF₃ that cause less inflammatory activities than PGE₂ (Perini et al., 2010; Andrade & Carmo, 2006; Garofolo & Petrilli, 2006; Sargi et al., 2012).

The increased consumption of LNA results in an increase in EPA and DHA phospholipids in the membrane of immune cells, enhancing the synthesis of eicosanoids with anti-inflammatory properties such as in PGE₃ and LTBS, which consequently decreases the synthesis of AA (Prini et al., 2010; Calder, 2007). Thus, importance is given to n-3 PUFAs because they act in inhibiting the synthesis of mediators derived from AA (Singer et al., 2008).

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the thermo-dimorphic fungus Paracoccidioides brasiliensis (Shikanai-Yassuda, Telles Filho, Mendes, Colombo, & Moretti, 2006; Palmeiro, Cherubini, & Yurgel, 2005). It is considered the most important fungal infection in Latin America, occurring in tropical and subtropical regions, being the eighth leading cause of death from chronic disease, including infectious and parasitic diseases, and the leading cause among the systemic mycoses in Brazil (Palmeiro et al., 2005; Bittencourt, Oliveira, & Coutinho, 2005).

The infection primarily involves the lungs, through inhalation of the fungus. It can subsequently spread to various organs and systems (Ramos-e-Silva & Saraiva, 2008; Gonzalez, Aristizábal, Gómez, Restrepo, & Cano, 2004). After infection, there are usually no immediate signs or symptoms of the disease, but the host develops a specific immune response against fungal antigens (Calich et al., 2008; Souto et al., 2000). PCM provides a range of clinical presentations, ranging from benign and localized to systemic and lethal disease, depending on the degree of depression of cellular immunity (Pedroso, Vilela, Pedroso, & Teixeira, 2009; Nascimento, Calich, Rodriguez, & Russo, 2002).

Various studies show that a protective response against P. brasiliensis depends on the response of CD4⁺ T lymphocytes, producing mainly IFN-γ, which activates macrophages, which are the main line of defense against P. brasiliensis in lung tissue. P. brasiliensis induces an inflammatory response that leads to the formation of granulomas containing a compact collection of cells of the mononuclear phagocytic system, which is the location of the primary lesion in PCM in order to contain and destroy the fungi and prevent its spread. Macrophages and CD4⁺ T lymphocytes assume central importance in the morphogenesis of the inflammatory process and the synergistic action of these cells is important in the formation and modulation of the granulomas (Rusas et al., 2009; Sadahiro, Diogo, Oshiro, & Shikanai-Yasuda, 2007; Arruda et al., 2002; Romano, Mendes-Giannini, Duarte, & Benard, 2002; Fortes, Kurokawa, Marques, Miot, & Marques, 2011). The CD8 lymphocytes may play an important role in the pathogenesis of pulmonary PCM, because these cells were found in patients with PCM along with pro-inflammatory cytokines produced by alveolar macrophages and specific P. brasiliensis antibodies (Chiarella et al., 2007; Fornazim et al., 2003).

This study aimed to evaluate the proliferative activity of splenic lymphocytes from mice previously infected with P. brasiliensis and fed a diet supplemented with flaxseed oil, a source of omega-3, which has demonstrated benefits mainly in inflammatory and immune response.

Material and methods

Fungal isolate

An isolate of P. brasiliensis (Pb18), cultivated in Fava Netto’s medium and kept at 35°C for 7 days was used in this study (Fava-Netto, Vegas, Sciannamea, & Guarnieri, 1969). The inoculums for infection in mice were prepared according to the method described in Sargi et al. (2013). The concentration of the cells was adjusted to 2 × 10⁶ fungi mL⁻¹ in a Neubauer chamber. Cell viability was determined by the Janus green dye exclusion method and was more than 95%.

Animals and infection of the animals with p. brasiliensis

All procedures involving the use of animals were approved by the Ethics Committee for Animal Experimentation of the State University of Maringa under protocol 006/2011. Groups of eight male Swiss mice, each aged 4 weeks old, were used. The animals were distributed into four groups of eight, and were inoculated intraperitoneally with a volume of 0.1 mL of a fungal suspension containing 2 × 10⁶ yeasts form cells of Pb18. The groups were divided as follows: control group uninfected (CGUI), control group infected (CGI), flaxseed group uninfected (FlaxGUI) and flaxseed group infected.
Diet

The diet was nutritionally complete and isocaloric and was formulated according to recommendations of the National Research Council (NRC, 1995). It was supplemented with 3% of flaxseed oil. The CGs received commercial feed. The composition of the experimental diet, expressed in percentages, was follows: corn, 45; soybean, 37.4; wheat, 6.9; flaxseed oil, 3; phosphate dicalcium, 2.5; calcium, 0.3; salt, 0.3; premix, 4.5. The flaxseed-enriched diet was prepared in bulk, separated into daily portions, vacuum packed and stored at 4°C. The total lipid content and fatty acid composition of the total lipids were monitored in the freshly prepared diets.

Isolation of mouse splenic lymphocytes and assessment of lymphoproliferative activity

The mice were sacrificed by cervical dislocation under anesthesia following animal ethics guidelines. The spleen was aseptically removed and then the lymphocytes were isolated from the spleen under aseptic conditions. Lymphocyte preparations with more than 90% viability (as assessed by the trypan blue dye exclusion test) were suspended in complete RPMI 1640 medium containing 10% fetal bovine serum. The cell count was adjusted to contain 2 × 10^7 lymphocytes mL^-1 and 100 μL of cell suspension was plated onto 96-well flat-bottom tissue culture plates and incubated for 2 hours at 37°C in 5% CO₂ and 95% air. Subsequently, the cultures were stimulated with PHA (1%) and then incubated for 24 hours at 37°C in 5% CO₂ and 95% air. Briefly, cells were incubated for 2 hours with 5 mg mL^-1 of MTT (Sigma-Aldrich), dissolved in phosphate-buffered saline. This was followed by addition of 100 μL of dimethyl sulfoxide and gentle shaking for 10 minutes so that complete dissolution was achieved. Absorbance was recorded at 570 nm using the microplate reader.

Statistical analysis

Data were analyzed by Mann-Whitney test after one-way ANOVA test using Statistic 8.0 software (StatSoft, 2007), and the differences were considered significant at p < 0.05.

Results and discussion

In all periods of the experiment (1st, 4th and 8th week) proliferative activity was observed in response to stimulation with PHA when comparing the groups CGUI, CGI, FlaxGUI and FlaxGI with the CG without PHA. In the 1st week (Figure 1) there was a decrease significant in lymphoproliferative activity of the group uninfected and fed a diet enriched with flaxseed oil (FlaxGUI), with respect to all experimental groups, including the CG without PHA (p = 0.002).

In the 4th week (Figure 2), an increase in cell proliferation in the animals of the FlaxGI group, compared to CGI and FlaxGUI (p = 0.006 and p = 0.002, respectively) was found.
In the 8th week (Figure 3), a decrease of lymphoproliferative activity of the FlaxGUI group compared to the CGUI group ($p = 0.025$) and an increase in the FlaxGI group compared to the FlaxGUI group ($p = 0.049$) were found.

Figure 3. Absorbance values of lymphocyte proliferation in the eighth week of infection: CG without PHA = control group of lymphocyte proliferation. CGUI = uninfected group fed commercial feed. CGI = infected group fed commercial feed. FlaxGUI = uninfected group fed an enriched-flaxseed oil diet. FlaxGI = infected group fed an enriched-flaxseed oil diet. * $p < 0.05$ CGUI versus FlaxGUI. # $p < 0.05$ FlaxGUI versus FlaxGI.

This study investigated the effect of flaxseed oil, a source of n-3 PUFAs on the proliferative activity of splenic lymphocytes during experimental infection with *P. brasiliensis*. In recent years, n-3 PUFAs have attracted much attention because their ability to modulate the immune system has been demonstrated (Hubbard et al., 1994; Andrade & Carmo, 2006; Garofolo & Petrilli, 2006; Calder, 2007).

The mechanism of action of n-3 PUFAs is due to the antagonism of eicosanoids derived from AA, i.e. these fatty acids, through EPA, participate in the generation of less potent and anti-inflammatory eicosanoids (Moreira, Dias-Melicio, Peraçoli, Calvi, & Soares, 2008; Bernard et al., 2001). Analyzing only the groups of animals fed commercial food (CGUI and CGI), there were no significant differences between them during all periods of the experiment. But among the groups fed an enriched-flaxseed oil diet (FlaxGUI and FlaxGI), there were significant differences in all weeks evaluated, demonstrating that an interaction between the n-3 PUFAs and the infection could be occurring.

Experimental models show that the proliferative response of lymphocytes tends to diminish in animals fed diets rich in n-3 fatty acids and NK cells show decreased phagocytosis and damage, responses occurring in immediate response to input of the fungus (Pompeia et al., 2000). Therefore, we suggest, in line with our results, that n-3 PUFAs decrease lymphoproliferative activity in the 1st week of the experiment, yet this activity resumed from the 4th week onward. It is assumed that recovery was due to an adaptation of the animal’s body to this kind of food, since functional foods have an action with continuous use in the diet. In this sense, we can also observe that the infection itself did not affect cell proliferation in the early phase of the infection, but in the 8th week of the experiment, an increase in the proliferation of lymphocytes was seen, which may be due to the balance achieved between immune and inflammatory intermediaries not observed in susceptible animals with experimental PCM, as in the case of Swiss mice (Almeida et al., 1998).

The CGI and FlaxGI groups showed no significant difference in lymphocyte proliferation at all times, with the exception of the 4th week in which there was a significant increase in proliferation of the FlaxGI group. PGE$_2$ and LTB4 are synthesized from AA and constitute potent immune mediators, the most important being PGE$_2$, since it has an immunosuppressive effect which inhibits lymphocyte proliferation and NK cells (Garofolo & Petrilli, 2006). In humans and experimental animals, supplementation with n-3 PUFAs establishes an extracellular AA in competition with the 5-LOX pathway, suppressing the formation of mediators such as PGE$_2$ (Chiarella et al., 2007; Bernard et al., 2001; Calder et al., 2009; Calder, 2006). Thus, it is expected that in this situation the animals fed the n-3 PUFAs produced a smaller amount of PGE$_2$, thus promoting lymphocyte activity when challenged by the fungus. However, this was only observed in the 4th week of infection, which can be explained due to the fact that the interaction between the fungus and the host response to this might reflect a further strength of the host to resisting infection. This host response, from the 4th week, might also be involved in the decrease of PGE$_2$. It is known that PGE$_2$ is produced by *P. brasiliensis* and is also capable of stimulating synthesis by the host early in infection (Biondo, Dias-Melicio, Bordon-Graciani, Acorci-Valério, & Soares, 2010), but over time the n-3 PUFAs can act to reduce numbers of this metabolite host and also could inhibit the synthesis of PGE$_2$ by the fungus, thus promoting lymphocyte proliferation responses later. During this period, we found that infected animals not receiving a flaxseed-enriched diet showed a significantly lower response, suggesting that n-3 PUFAs have a stimulatory role in respect to lymphocytes in the 4th week of infection.
The level of proliferation continued until the 8th week, a later stage of infection, which is to be expected considering Swiss mice can be cured of infection over time, which can be confirmed by the response observed in the CGI group, which had the same level of cell proliferation during the 8th week of the experiment.

Studies have reported on the influence of n-3 PUFAs in reducing cell proliferation. In analyzing our results, the reductive influence of n-3 PUFAs on the lymphoproliferative response early in infection corroborates with the literature. However, in later stages, the presence of n-3 PUFAs in diets seems to have influenced the increase of lymphocyte proliferation. There are few studies in the literature which relate the dynamics of the effect of n-3 PUFAs in the host response in infectious diseases. Due to the importance of the impact and concern about PCM at the levels of state and country, further research is necessary for us to understand the real role of n-3 PUFAs in the improvement of the host response during PCM.

**Conclusion**

So, according to the data presented, the findings indicated that the n-3 PUFAs reduced lymphoproliferative activity in the initial stage of experimental murine PCM and increased the proliferation of lymphocytes in the later phase of infection. However, more studies are necessary to establish this point.

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