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Experimental pulmonary paracoccidioidomycosis progression after p2x7 receptor inhibition

Análise da evolução patológica da paracoccidioidomicose pulmonar experimental após inibição do receptor p2x7

Análisis de la evolución patológica de la paracoccidioidomicosis pulmonar experimental tras la inhibición del receptor p2x7

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Abstract
Objective: To evaluate the effects of P2X7 receptor inhibition by Brilliant Blue G dye (BBG) in an experimental mode of pulmonary paracoccidioidomycosis. Methodology: Thirty male rats were divided into five groups: absolute control group, negative control group, experimental group 50, experimental group 100, and experimental group 150. The last four groups underwent experimental induction of pulmonary paracoccidioidomycosis; however, only the brilliant blue G experimental groups 50, 100, and 150 were administered BBG intraperitoneally every 48 h at doses of 50, 100, and 150 mg/kg respectively, to inhibit P2X7 receptor activity. To assess the effects of this treatment, lung tissue was analyzed histologically and the levels of the anti-gp43 antibody were measured by enzyme-linked immunosorbent assay. Results and conclusion: Lung biopsies of the animals treated with BBG exhibited a significant reduction in the number and area of granulomas with lower levels of anti-gp43 antibodies relative to those in the untreated group. Treatment with BBG reduced the number and area of granulomas in the lung tissue and levels of anti-gp43 antibodies against Paracoccidioides brasiliensis, thereby mitigating pulmonary paracoccidioidomycosis in the experimental model.
Keywords: Paracoccidioides brasiliensis; Paracoccidioidomycosis; Lung Diseases Fungal; Purinergic P2X7 Receptor.

Resumo
Objetivo: Avaliar os efeitos da inibição do receptor P2X7, através da utilização do corante Brilliant Blue G, no modelo experimental de paracoccidioidomicose pulmonar. Metodologia: Foram utilizados 30 ratos machos, distribuídos em cinco grupos: Grupo controle absoluto, Grupo controle negativo, Grupo experimental 50, Grupo experimental 100, grupo experimental 150. Os quatro últimos grupos sofreram indução do modelo experimental de Paracoccidioidomicose pulmonar, porém, apenas os Brilliant Blue G dos grupos experimental 50, 100 e 150 receberam aplicação intraperitoneal de BBG a cada 48 horas, nas doses de 50, 100 e 150 mg/kg respectivamente, para a inibição da atividade do receptor P2X7. Para analisar os efeitos da terapia proposta foi avaliado histologicamente o tecido pulmonar dos ratos e dosado o anticorpo anti-Gp 43 através do método ELISA. Resultados: As biópsias pulmonares dos animais tratados com Brilliant Blue G apresentaram diminuição significativa do número e área de granulomas em relação ao grupo não tratado e menor titulação de anticorpos anti-Gp43 nos grupos que receberam o tratamento com BBG. Conclusões: O tratamento com Brilliant Blue G foi capaz de reduzir o número e área de

granulomas nos tecidos pulmonares e os níveis do anticorpo anti-Gp43 de Pb, atenuando a Paracoccidioidomicose pulmonar no modelo experimental utilizado.
Palavras-chave: Paracoccidioides brasiliensis; Paracoccidioidomicose; Micose pulmonar; Receptor Purinérgico P2X7.

Resumen

Objetivo: Evaluar los efectos de la inhibición del receptor P2X7, mediante el uso del colorante Brilliant Blue G, en el modelo experimental de paracoccidioidomicosis pulmonar. Metodología: Treinta ratas macho fueron divididas en cinco grupos: grupo control absoluto, grupo control negativo, grupo experimental 50, grupo experimental 100, grupo experimental 150. A los últimos cuatro grupos se les realizó inducción del modelo experimental de paracoccidioidomicosis pulmonar, sin embargo, solo el Brilliant Blue G de los grupos experimentales 50, 100 y 150 recibieron aplicación intraperitoneal de BBG cada 48 horas, en dosis de 50, 100 y 150 mg/kg, respectivamente, para inhibir la actividad del receptor P2X7. Para analizar los efectos de la terapia propuesta, se evaluó histológicamente el tejido pulmonar de las ratas y se midió el anticuerpo anti-Gp 43 mediante el método ELISA. Resultados: Las biopsias pulmonares de animales tratados con Brilliant Blue G mostraron una disminución significativa en el número y área de granulomas en relación al grupo no tratado y menor título de anticuerpos anti-Gp43 en los grupos que recibieron tratamiento con BBG. Conclusiones: El tratamiento con Azul Brillante G logró reducir el número y área de granulomas en los tejidos pulmonares y los niveles de anticuerpo anti-Pb Gp43, atenuando la Paracoccidioidomicosis pulmonar en el modelo experimental utilizado.
Palabras clave: Paracoccidioides brasiliensis; Paracoccidioidomicosis; Enfermedades Pulmonares Fúngicas; Receptor Purinérgicos P2X7.

1. Introduction

Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America and is caused by the fungus of the genus Paracoccidioides spp., which includes Paracoccidioides brasiliensis (Pb) and Paracoccidioides lutzii. Approximately 80% of cases are reported in Brazil and the remaining cases occur in countries such as Venezuela, Colombia, Ecuador, and Argentina (Restrepo et al., 2011; Colombo et al, 2011). PCM is the eighth leading cause of death from chronic infectious and parasitic diseases in Brazil. Most cases are concentrated in states of the South and Southeast regions of the country. The state of Paraná accounts for 53.2% of deaths related to PCM in the South region (Restrepo et al., 2011; Lacaz, 2002).

A survey showed that 2,169 cases of PCM were reported between 1966 and 2009 in the South region, with a mean of 140.6 cases per year (Martinez, 2015). In the Itaipu reservoir region, which is to the west of Paraná, 102 new cases of this disease were reported over a period of only 18 months (Loth et al., 2011).

It has been estimated that the number of individuals affected by PCM is higher than that published. Thus, this mycosis is a neglected disease, and its damages may be even greater than realized (Martinez, 2010).

Paracoccidioides brasiliensis is a thermally dimorphic fungus that grows as mycelium in the environment at temperatures of 24–25°C or as yeast in vitro and in warm-blooded animals at temperatures of 36–37°C (Wanke & Londero, 1994; Theodoro et al., 2005; San-Blas & Niño-Vega, 2008).

PCM is a serious public health problem because of its debilitating potential. Moreover, it predominantly affects male individuals in the most productive years of their life (Martinez, 2010; Bellisimo-Rodrigues et al., 2011). Approximately 90% of PCM cases occur in individuals aged 30–50 years, leading to high social and economic costs (Wanke & Londero, 1994; Negroni, 1993; Mota, 1996). Some factors worsen the problem of PCM for public health, such as delayed diagnosis, undetected disease progression, and difficulty in accessing healthcare services, in addition to its similarity to other granulomatous diseases (Marques, 2012; Martinez & Moya, 2009).

Infection occurs primarily by accidental inhalation of fungal propagules that lodge in the lung tissue; these spores may spread to any organ via the lymphatic or hematogenous route1. The organ most frequently involved is the lung, which is affected in as many as 90% of patients, resulting in granulomatous inflammation, fibrosis, and chronic pulmonary failure

(Shikanai-Yasuda et al., 2018; Shankar et al., 2011).

Both Pb and P. lutzii are sensitive to most systemic antifungal drugs (Shikanai-Yasuda et al., 2018), including sulfonamides, amphotericin B, and azole derivatives, which are currently available for treating PCM (Martinez et al., 2006). However, these long-term therapies lead to problems with patient adhesion (Travassos, Taborda, Colombo, 2008). Therefore, patients with PCM should be monitored until they are cured, but these patients remain at risk of fungal reactivation (Shikanai-Yasuda et al., 2018).

P2x7 receptors (P2x7r) are purinergic membrane receptors activated by ATP and are expressed in a variety of human cells and tissues such as the heart, liver, skeletal muscle, pancreas, monocytes, macrophages, T lymphocytes, and antigen-presenting cells (Jiang, 2009; Gavala et al., 2008; Bulanova et al., 2009; Geraghty et al., 2016; Abbracchio et al., 2009). These receptors are involved in several pathophysiological processes, including apoptosis, necrosis, inflammatory response, and elimination of infectious agents (Volonté et al., 2012).

Studies have shown that inhibition of P2x7r may lead to mitigation of diseases, such as tuberculosis, and decreased morbidity rates. Inactivation of this receptor reduces immune cell recruitment, thereby reducing the exacerbated inflammatory process and, in turn, tissue necrosis (Amaral et al., 2014; Monção-Ribeiro et al., 2014). Brilliant blue G (BBG) is a food dye used in biological tests that selectively blocks P2x7r, indicating its potential as a potent pharmacological agent (Soares-Bezerra et al., 2015; Sooyeon & Bruce, 2011; Bin Dayel et al., 2019)

Considering the high rates of treatment interruption, difficulties faced by patients with PCM, evidence in the literature demonstrating the beneficial effects of P2x7r inhibition against the disease processes, and low toxicity of BBG, studies are necessary to investigate the effects of this treatment on pulmonary PCM. Thus, the aim of the present study was to assess the effects of P2x7r inhibition by BBG in an experimental rat model of pulmonary paracoccidioidomycosis.

2. Methodology

The study was conducted in the Research Laboratory of the Western Paraná State University (Laboratório de pesquisa da Universidade Estadual do Oeste do Paraná) in Cascavel/PR, according to the principles stated in the Guide for Care and use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academic Science, Washington, DC (1996) and Ethical Principles in Animal Experimentation according to Law and the policies of the Brazilian College of Animal Experimentation (COBEA). The study was approved by the Animal Research Ethics Committee (CEUA) of UNIOESTE before starting the experiments.

The animals used in the study were 8-week-old male rats (n = 30) obtained from the central animal facility (Biotério Central) of Western Paraná State University (UNIOESTE). They were fed a standard diet of food pellets and water ad libitum and kept in standard boxes (four rats per box) under a light/dark cycle of 12 h and controlled temperature of 23°C. The rats were divided into five groups: absolute control group (ACG), which was not infected and did not receive any specific treatment; negative control group (NCG), which was induced with pulmonary paracoccidioidomycosis but did not receive any specific treatment for the disease and served as control for the groups treated with the P2X7 receptor inhibitor; and experimental groups EG50, EG100, and EG150, which were induced with pulmonary PCM and injected intraperitoneally every 48 h with BBG diluted in sterile saline solution at doses of 50, 100, and 150 mg/kg, respectively, starting on the seventh day after infection.

The experimental model of pulmonary paracoccidioidomycosis used in the NCG, EG50, EG100, and EG150 groups was induced by inoculation of a single dose of a cell suspension of P. brasiliensis at a concentration of 1 × 105 yeast in 0.1 mL phosphate-buffered saline, with a cell viability above 90%. The yeast suspension was administered directly into the animals' trachea by orotracheal intubation using an Abbocath peripheral venous catheter. Subsequently, the animals were manually

ventilated with a sphygmomanometer bulb connected to the Abbocath catheter to ensure dissemination of the cell suspension to all areas of the lung. The procedure was performed under anesthesia with ketamine and xylazine administered intraperitoneally (80 and 15 mg/kg of body weight, respectively).

The viability test was performed by removing an aliquot of each cell suspension to which trypan blue was added at a proportion of 1:1, and the cells were counted under a microscope. The samples with a cell viability higher than 90% were used for subsequent analysis.

The experiment was conducted over a period of 22 days, from the inoculation of fungus to animal sacrifice. During the first seven days after inducing the experimental model of pulmonary PCM, all groups were untreated. After the seventh day, the experimental groups EG50, EG100, and EG150 were administered BBG for 15 days. Twenty-four hours after administration of the last dose of BBG, all animals were sacrificed by decapitation under anesthesia with ketamine and xylazine at the doses described above.

Lung tissue was collected immediately after sacrifice for histological analysis. The samples were fixed and stained with hematoxylin/eosin (Junqueira & Junqueira, 1983) and with Grocott's methenamine silver stain (Grocott, 1955) to detect Pb.

Blood was collected and placed in appropriate plastic tubes for analysis by enzyme-linked immunosorbent assay (ELISA), which was performed according to Ramos et al. (2005). Ninety-six-well plates coated with purified antigen gp43 and secondary antibody (anti-IgG peroxidase, Sigma-Aldrich, St. Louis, MO, USA) were used. The assays were performed in triplicate and absorbance was read in a microplate reader at 492 nm.

Statistical analysis was performed by simple descriptive analysis, with the distribution frequency calculated to quantitatively assess the lung biopsies. Disease intensity was classified considering the following intensity of anatomopathological signs: absent (without anatomopathological signs), mild (discrete anatomopathological signs), moderate (diffuse anatomopathological signs), or severe (intense anatomopathological signs and even focal necrosis).

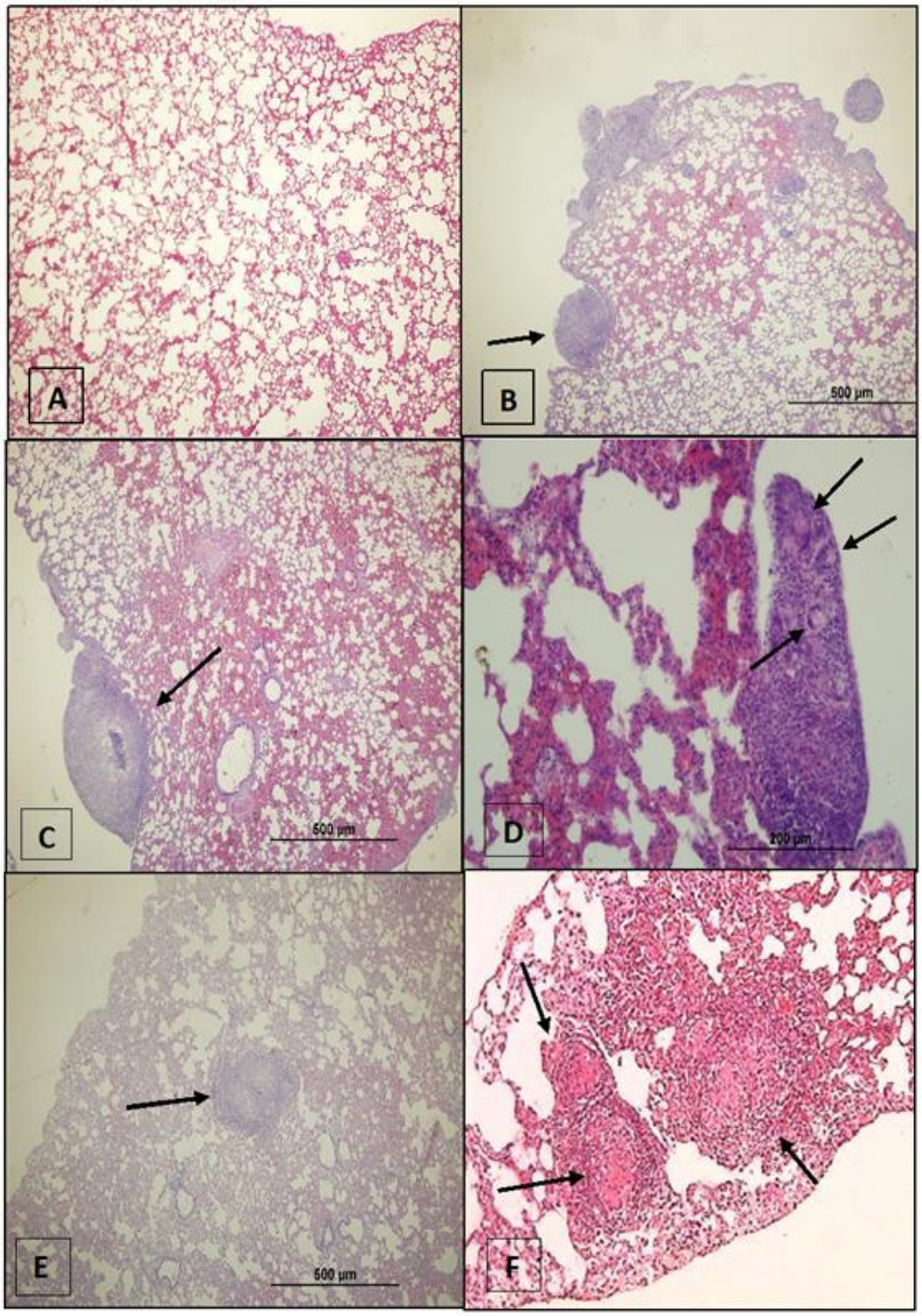
Analysis of variance was used to compare the results of the ELISA and morphometry analysis at a level of significance of 5% with α set to $p < 0.05$. Analyses were performed using the software GraphPad Prism, version 3.0 (GraphPad, Inc., La Jolla, CA, USA), Microsoft Office®, and Office Excel® software for Windows XP (Seattle, WA, USA).

3. Results

Qualitative analysis of the lung biopsies revealed intense tuberculoid granulomatous inflammation of the lung in 100% of the animals in the NCG, with diffuse inflammatory infiltrate and numerous epithelioid granulomas with different degrees of maturity. These granulomas contained Langhans giant cells with Pb inside in the form of yeast with multiple buds. Additionally, budding yeasts were observed in the periphery of the granulomas. Lung biopsies of this group showed features of pneumonia with intense and diffuse cellular infiltrate throughout the lung tissue.

The experimental groups treated with BBG exhibited discreet formation of foci of epithelioid granulomatous inflammation, particularly at the outer edges of the lung specimens. The granulomas were typical of pulmonary PCM and rare granulomas infiltrated the lung parenchyma. Yeast-like fungi with multiple budding containing giant cells were only observed inside these granulomas. There were no fungi outside the granuloma area and granulomas were predominantly organized. Animals in the ACG did not show signs of disease.

Figure 1 - Photomicrograph of histological samples of the lungs of the animals in the study groups.



Source: Authors.

Figure 1 – A –Normal structure with preserved alveoli, absence of inflammatory process in an animal in the absolute control group (staining with hematoxylin-eosin, HE, 4x). B – Presence of organized epithelioid granulomas at the edge of the lung, indicated by the arrows, and lung parenchyma with mild inflammatory infiltrate in an animal in the group treated with 50 mg/mL BBG (HE, 4x). C – Presence of isolated granuloma adhering to the outer edge of the lung of a rat treated with 50 mg/mL BBG, indicated by the arrow (HE, 4x). D – Small isolated epithelioid granuloma, at higher magnification, adhering to

the outer edge of the lung of a rat treated with 100 mg/mL BBG. Arrows indicate Langhans giant cells (HE, 20x). E - Small epithelioid granuloma inside the lung parenchyma of a rat treated with 150 mg/mL BBG, indicated by the arrow (HE, 4X). F – Shows the presence of several granulomas (arrows), in a histological section of the lung of a rat in the NCG (HE, 4x).

Quantitative analysis was conducted to assess the intensity of PCM in the lungs of rats and classification according to the absence or presence of histopathological signs of disease: absent (without histopathological signs), mild (mild histopathological signs), moderate (diffuse histopathological signs), and severe (intense histopathological signs and even focal necrosis). The data indicated that 100% of rats in the NCG had severe pulmonary disease, whereas all experimental groups treated with BBG exhibited attenuated anatomopathological signs and mild pulmonary disease (Figure 1).

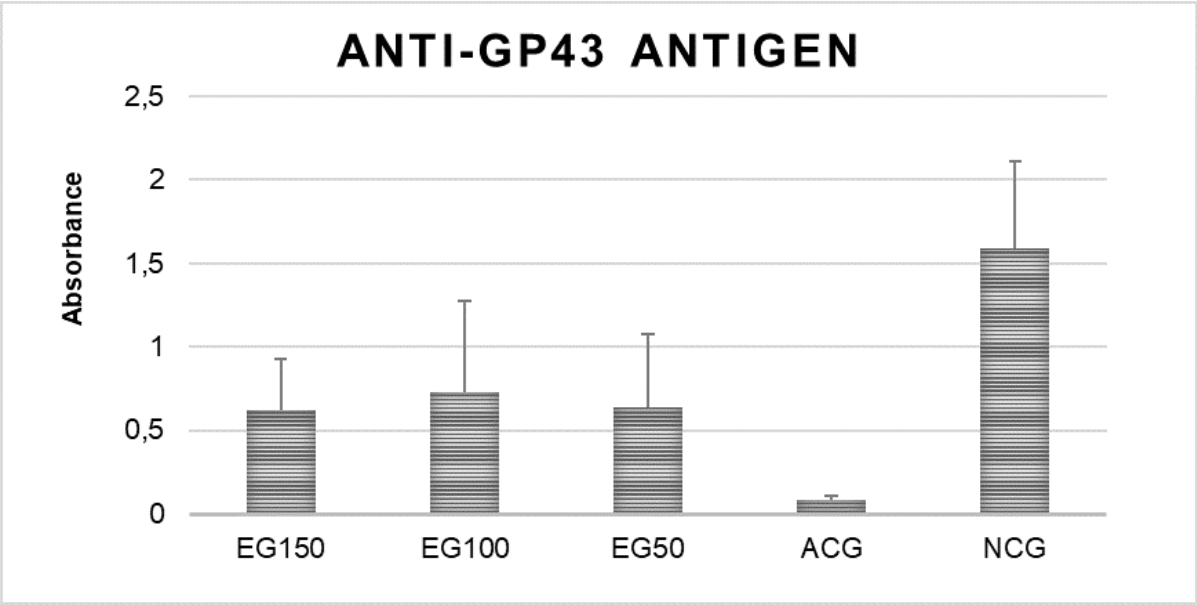
There was a significant difference in the number of granulomas between groups (p-value = 0.0001). The mean number of granulomas per area in the NCG was 16.5 (±3.45), whereas in the experimental groups this value was 2.1 (±0.5) for an analyzed area of the same size.

Neither histological qualitative analysis nor quantitative analysis revealed differences between the EG150, EG100, and EG150 groups. However, there were significant differences between the results obtained in the experimental groups and NCG.

Morphometric analysis indicated that the mean area of granulomas in the lungs of rats in the NCG was 10.741 (±2.456) µm2. In the experimental groups, the mean area was 6.259 (±0.975) µm2. Moreover, ANOVA indicated a significant difference in granuloma size between the NCG and experimental groups (p-value = 0.001).

Titration of anti-gp43 antibodies by ELISA showed no differences in the production of specific antibodies between the experimental groups. However, comparison of the experimental groups and NCG showed significant differences (p-value = 0.0001), with lower values in the groups treated with BBG (Figure 2).

Figure 2 - Titration of anti-gp43 antibodies by ELISA.



Source: Authors.

Figure 2 - There were no differences in the production of anti-gp43 antibodies against Pb between experimental groups. The comparison between the experimental groups and NCG showed significant differences, with lower values in the groups treated with BBG.

4. Discussion

The aim of the present study was to assess the effects of P2x7r inhibition by BBG in an experimental model of pulmonary PCM. The primary objective was to investigate the potential of this treatment as an adjuvant to treatments already in use. The current treatment of PCM is based on antifungal agents that require long periods of administration to yield a cure (Shikanai-Yasuda et al., 2018).

In the present study, the groups of animals treated with BBG exhibited pulmonary PCM mitigation, as shown by the lower titer of anti-gp43 antibodies against Pb in the ELISA. Additionally, morphometry analysis revealed significant differences in the mean number and area of granulomas between the study groups.

This is the first study to use BBG as a P2x7r antagonist in pulmonary PCM. Moreover, although the efficacy of BBG has been reported previously, studies using P2x7r antagonism in pulmonary disorders are rare. Monção-Ribeiro et al. (2014) used a murine model of lung silicosis in which animals were treated with 45 mg/kg of BBG twice/weekly for two weeks and demonstrated a reduced lung fibrosis and inflammation markers. These results are similar to those obtained in the present study, in which the groups treated with BBG also showed significant disease mitigation, as demonstrated by the typical anatopathological signs of PCM: lower titer of anti-gp43 antibodies against Pb and decreased number and area of granulomas in the treated groups.

Wang et al. (2015) conducted a study in which rats with acute lung injury induced by lipopolysaccharide were treated with intraperitoneal BBG at a dose of 45.5 mg/kg every 48 h, as well as with another inhibitor, A438079, every 24 h. These authors observed that both P2x7r blockers significantly reduced macrophage infiltrate and lung injury in the treated animals. Notably, the BBG treatment regimen used in the study was similar to that used in the present study, both with regard to doses and intervals between doses. Thus, the results presented by Wang et al., including reduced signs of inflammation, are similar to our results.

A study conducted by Kolluputti et al., (2010) indicated that P2x7r inhibition in experimentally induced pulmonary injury reduced inflammasome activation and the synthesis of proinflammatory cytokines and formation of pores in the cell membrane of alveolar macrophages, thereby providing a strategy for protecting lung tissue against cellular injury. Lucatelli et al. (2011) also reported the beneficial effects of P2x7r inhibition in animals with lung inflammation induced by exposure to tobacco smoke. These authors observed that inhibition of P2x7r in this experimental model of injury mitigated and even prevented acute lung injury. They observed the same effects after chronic exposure to tobacco smoke.

Considering the high inflammatory potential of infection caused by Pb (Fortes et al., 2011), results of lung biopsies of the study animals, and data from the studies reviewed in the discussion, the PCM mitigation and decreased tissue destruction found in the groups treated with BBG may have occurred because of a reduction in inflammatory activity caused by P2x7r inhibition.

Other authors used the BBG dye in experimental models of diseases with an inflammatory pattern in other tissues, such as in experimental ulcerative colitis, in which the use of the BBG antagonist was effective in the recovery of enteric neurons (Evangelinellis, 2019) and in experimental parkinsonism, where blockade of the P2X7 receptors with BBG can prevent present motor and non-motor deficits through mechanisms that involve the control of neuroinflammation and dopaminergic degeneration (Oliveira et al., 2021).

Other studies investigated the effects of inhibiting this receptor using animals genetically deficient in P2x7r. Amaral et al. (2014) investigated the effect of P2x7r inhibition using knockout mice and hypervirulent strains of tuberculosis, a disease that involves lung tissue in a similar manner as PCM. The authors concluded that the knockout animals had a 10-fold lower bacillary load, lower dissemination of the disease to other organs such as the liver and spleen, and less lung tissue destruction than control animals.

Riteau et al. (2010) used a murine model of induced pulmonary inflammation and fibrosis in animals genetically deficient in P2x7r. These authors observed reduced signs of fibrosis and lung remodeling in the knockout animals 14 days after model induction. Although different methods of P2x7r inhibition were used in the studies, they confirm the current results of mitigation of the disease, with lower levels of fungi observed inside granulomas and lower levels of the anti-gp43 antibodies against Pb. Thus, the proposed treatment of inhibiting P2x7r with BBG at different doses minimized the effects of pulmonary PCM in rats in the experimental groups. These findings are useful for future studies focusing on the search for new treatments for PCM.

5. Conclusion

Inhibition of the P2x7 receptor using BBG as antagonist led to a decrease in inflammation, number and area of granulomas, and titer of anti-gp43 antibody against Pb at all tested doses. These results are promising and warrant further investigation. It is important to emphasize that the present study is a pioneering experiment in the use of BBG in a model of pulmonary PCM, thus, other experimental designs with different doses of the dye, at different times, are necessary, in addition to comparing the results of this antagonist with other P2X7 receptor antagonists in pulmonary PCM.

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