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Avaliação de terapias antifibróticas associadas aos antifúngicos itraconazol e cotrimoxazol em modelo murino de paracoccidioidomicose pulmonar

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestra em Doenças Tropicais.

Orientador: Prof. Dr. James Venturini

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Palavras-chave: Antifibrótico; Azitromicina; Fibrose pulmonar; Pentoxifilina; Talidomida.

Dedicatória

Dedicatória

Aos pacientes com paracoccidioidomicose,
na esperança deste trabalho
contribuir verdadeiramente em suas vidas.

*À minha prima Giseli (**in memoriam**),*
a qual foi a primeira a me mostrar as belezas e
as tristezas de uma pesquisa científica.

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Agradecimentos

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"Trabalho, aquilo que dignifica o homem!
Em qualquer campo de ação, a luta deve ser sempre justa e
em prol do bem comum."
(Dirsomar Chaves)

Resumo

Resumo

FINATO, A. C. **Avaliação de terapias antifibróticas associadas aos antifúngicos itraconazol e cotrimoxazol em modelo murino de paracoccidioidomicose pulmonar.** Dissertação (Mestrado) – Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, 2017.

Paracoccidioidomicose (PCM) é uma micose sistêmica causada por fungos do gênero *Paracoccidioides*; suas principais formas clínicas são aguda/subaguda, crônica e residual. A PCM é uma doença restrita a países da América Latina com maior incidência no Brasil, especialmente entre os trabalhadores rurais. A maioria dos pacientes com a forma crônica da doença, mesmo após tratamento eficaz, apresentam sequelas, incluindo fibrose pulmonar e adrenal. Os problemas sociais, econômicos e psicológicos desencadeados pela fibrose pulmonar são subestimados; além disso, a fibrose na PCM permanece negligenciada, uma vez que não há tratamento. Dessa forma, o estudo teve por objetivo investigar a influência de drogas com potencial antifibrótico (pentoxifilina - PTX, azitromicina - AZT e talidomida - Thal) associadas aos tratamentos antifúngicos com itraconazol - ITC e cotrimoxazol - CMX em modelo murino de PCM pulmonar. Para tanto, camundongos BALB/c machos foram inoculados com leveduras do isolado 326 de *P. brasiliensis* e após 8 semanas de infecção foi dado início aos esquemas terapêuticos: PTX/ITC, PTX/CMX, AZT/ITC, AZT/CMX, Thal/ITC e Thal/CMX. Após 8 semanas de tratamento, os animais foram eutanasiados a fim de se avaliar a deposição de fibras colágenas, produção de hidroxiprolina, recuperação de fungos viáveis e a porcentagem das áreas com lesão nos pulmões e peso corporal. Visando identificar os mecanismos envolvidos foi avaliada a produção de TGF-β1, CCL3, IFN-γ, TNF-α, IL-10, VEGF, IL-6, IL-1β, IL-17 e IL-2 no homogenato dos pulmões. Nossos achados revelaram que os camundongos infectados tratados com PTX/ITC mostraram redução nos níveis de hidroxiprolina associada a menor produção de IL-6, IL-17 e TGF-β1 e níveis mais elevados de IL-10 em comparação ao tratamento apenas com ITC. De forma semelhante, os camundongos infectados tratados com AZT/CMX apresentaram menos fibrose pulmonar associada a níveis reduzidos de TGF-β1 e aumento de TNF-α e IL-10 do que os camundongos infectados tratados com CMX. Ambos os grupos em que os camundongos foram tratados com ITC/Thal e

CMX/Thal mostraram perda de peso intensa, aumento da deposição de fibras reticulares, altos níveis de CCL3, IFN-γ e VEGF e níveis diminuídos de IL-6, IL-1β, IL-17 e TGF-β1 em relação aos camundongos infectados tratados com ITC ou CMX. Dessa forma, nossos achados reforçam o papel antifibrótico da PTX na PCM pulmonar quando associado ao ITC. Além disso, nossos resultados apontaram a AZT como candidata a droga antifibrótica em associação com CMX e a ineficácia da Thal, no tratamento da PCM pulmonar.

Palavras-chave: Antifibróticos; Azitromicina; Fibrose pulmonar; Pentoxifilina; Talidomida.

Abstract

Abstract

FINATO, A. C. **Evaluation of antifibrotic therapies associated with itraconazole and cotrimoxazole in a murine model of pulmonary paracoccidioidomycosis.** Thesis (Master) – Faculty of Medicine of Botucatu, Universidade Estadual Paulista, Botucatu, 2017.

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by fungi of *Paracoccidioides* genus; the main clinical forms are acute/subacute, chronic and residual. PCM is a disease restricted to Latin American countries with a higher incidence in Brazil, especially among rural workers. Most patients with the chronic form, even after effective treatment, present sequelae, including pulmonary and adrenal fibrosis. The social, economic and psychological problems triggered by pulmonary sequels are underestimated. In addition, fibrosis in PCM remains neglected, since there is no treatment. The aim of this study was to investigate the influence of antifibrotic drugs (pentoxifylline - PTX, azithromycin - AZT and thalidomide - Thal) associated with antifungal treatments with itraconazole - ITC and cotrimoxazole - CMX in a murine model of pulmonary PCM. Male BALB/c mice were inoculated with *P. brasiliensis* “isolated 326” and after 8 weeks of infection the treatment were started: PTX/ITC, PTX/CMX, AZT/ITC, AZT/CMX, Thal/ITC and Thal/CMX. After 8 weeks of treatment, the mice were euthanized in order to evaluate the deposition of collagen fibers, hydroxyproline production, recovery of viable fungi and the percentage of areas with injury in lung and body weight. In order to identify the mechanisms involved, the production of TGF-β1, CCL3, IFN-γ, TNF-α, IL-10, VEGF, IL-6, IL-1β, IL-17 and IL-2 in the lung homogenate was evaluated. Our findings revealed that infected mice treated with PTX/ITC showed a reduction in hydroxyproline levels associated with lower production of IL-6, IL-17 and TGF-β1 and higher levels of IL-10 compared to treatment with ITC. Similarly, infected mice treated with AZT/CMX had less lung fibrosis associated with reduced levels of TGF-β1 and increased TNF-α and IL-10 than infected mice treated with CMX. Both groups in which the mice were treated with Thal/ITC and Tha/CMX showed intense weight loss, increased deposition of reticulin fiber, high levels of CCL3, IFN-γ and VEGF and decreased levels of IL-6, IL- 1β, IL-17 and TGF-β1 than to the infected mice treated with ITC or CMX. Thus, our findings reinforce the antifibrotic role of PTX in pulmonary PCM when associated with ITC. Besides, our results point out the AZT as a candidate for antifibrotic drug in association with CMX and the inefficacy of Thal in

the treatment of pulmonary PCM.

Keywords: Antifibrotic; Azithromycin; Pentoxifylline; Pulmonary fibrosis; Thalidomide.

Sumário

Sumário

1. **Introdução**.....21
2. **Objetivo geral**.....26
   2.1. Objetivos específicos.....26
3. **Referências bibliográficas**.....27
4. **Manuscrito**.....33
5. **Conclusões**.....63

Introdução

1. Introdução

1.1. Aspectos da paracoccidioidomicose e de seu agente etiológico.

Descrita em 1908 por Adolfo Lutz, a paracoccidioidomicose (PCM) é uma micose sistêmica, granulomatosa causada por fungos termodimórficos do gênero *Paracoccidioides*(1,2).

O gênero *Paracoccidioides* é composto por duas espécies, o *P. brasiliensis* (Pb) e o *P. lutzii*. O *P. lutzii* é um grupo monofilético e recombinante encontrado no centro, sudoeste e norte do Brasil e do Equador(1), já o Pb é um grupo monofilético composto por espécies crípticas classificadas recentemente em S1a, S1b, PS2, PS3 e PS4(3). As espécies crípticas S1a e S1b estão associadas à maioria dos casos de PCM e está amplamente distribuída na América do Sul(1,3–5). A espécie críptica PS2 foi identificada até o momento no Brasil e na Venezuela, a PS3 é encontrada principalmente em regiões endêmicas da Colômbia(1,4) e a PS4 foi descrita na Venezuela(5).

Apesar de ter sido isolado algumas vezes do solo, o nicho ecológico dos fungos *Paracoccidioides* spp. ainda é pouco conhecido. Os tatus da espécie *Dasypus novemcintus* (popularmente conhecidos como tatu de nove bandas ou tatu galinha) são reservatórios naturais desse fungo(6–8).

A PCM é uma doença endêmica no Brasil, geograficamente restrita à América Latina, do México à Argentina, apresenta incidência de 1-3 casos por 100.000 habitantes e a maioria dos indivíduos afetados são trabalhadores rurais do sexo masculino(9). A doença pode comprometer qualquer órgão, com predomínio de pulmões, órgãos ricos em células do sistema fagocítico mononuclear, mucosa das vias aerodigestivas superiores, pele e adrenais(10).

As principais formas clínicas da doença são: a) a forma aguda/sub-aguda, também chamada juvenil que em geral compromete crianças, adolescentes e adultos jovens, apresenta história clínica de curta duração (mediana de dois meses) e exibe manifestações clínicas compatíveis com o comprometimento de órgãos do sistema fagocítico mononuclear; b) a forma crônica, em geral compromete adultos com mais de 30 anos de idade, que apresentam história clínica de longa duração (em geral acima de seis meses), acometendo pulmões e mucosa das vias aerodigestivas superiores com grande frequência; c) forma residual, caracterizada

pelas sequelas observadas após tratamento, em especial nos pulmões, laringe e adrenais(11).

Até a década de 1940 não havia uma droga padrão utilizada no tratamento da PCM, assim, apresentando evolução fatal. Em 1958, a anfotericina B, um potente antifúngico de amplo espectro, foi introduzido no tratamento demonstrando ser eficaz, entretanto a administração do medicamento requer via intravenosa e hospitalização do paciente, além de demonstrar efeitos adversos(12). Assim, no início da década de 1970, demonstrando menores efeitos adversos e de fácil administração, a associação sulfametoxazol-trimetoprima (cotrimoxazol – CMX) se torna amplamente utilizada no tratamento da doença(13). Ainda em busca de fármacos, no final da década 1980 surgiram os azólicos. O itraconazol (ITC) mostrou-se muito eficaz e seguro no tratamento(14–16) e foi considerado a droga de primeira escolha para o tratamento da PCM(17).

No Brasil, o CMX possui distribuição gratuita pelo Sistema Único de Saúde (SUS) e, por isso, continua sendo amplamente empregado no tratamento da PCM.

1.2. PCM forma crônica (FC) e fibrose pulmonar

Mesmo após tratamento antifúngico eficaz, a avaliação clínica e radiológica de pacientes com a FC revela a presença de fibrose nos diferentes órgãos comprometidos. Os pulmões, além da fibrose, também exibem enfisema - possivelmente pela elevada incidência de tabagismo nesses pacientes(18–21). Santos e colaboradores (2003)(21) consideraram o tabagismo como fator de risco para a PCM. Nesse estudo foi observado que fumantes possuem cerca de 14 vezes mais chances de desenvolver a doença e, adicionalmente, cerca de 3 vezes mais chances em indivíduos etilistas.

Os achados necroscópicos de pacientes com PCM revelam que a fibrose pulmonar é caracterizada por extensas áreas de depósito de colágeno próximas à região hilar, envolvendo outras estruturas como linfonodos, brônquios e artérias. As fibras colágenas se encontram na periferia dos granulomas e se estendem a brônquios e vasos sanguíneos próximos. A proliferação de fibras reticulares (colágeno III) também ocorre no septo alveolar, inclusive em áreas distantes do processo granulomatoso, sugerindo que o próprio fungo e/ou seus antígenos possam atuar diretamente sobre a proliferação de fibras reticulares nos alvéolos

pulmonares(22).

Essas sequelas levam ao comprometimento do órgão e consequentemente a incapacitação do paciente, levando a problemas econômicos e sociais, tendo em vista, que os pacientes se encontram na fase mais produtiva profissionalmente de suas vidas (adultos a partir de 30 anos). A incapacitação nesses pacientes, visto que comumente são tabagistas e etilistas, pode contribuir para o agravamento dessas condições e consequentemente levar a sintomas depressivos(23).

Apesar da sua importância, poucos estudos têm focado especificamente na fibrogênese pulmonar que ocorre na PCM. Cock e colaboradores (2000)(24) demonstraram em modelo experimental murino que esse processo é precoce, onde os animais após 1 semana de infecção apresentaram início do processo granulomatoso e a partir da 4ª de infecção apresentaram granulomas com aumento da deposição de fibras de colágeno tipo III. Araújo (2011)(25), observou em estudos necroscópicos presença da fibrose em pacientes que não receberam tratamento antifúngico, demonstrando assim, que os pacientes com PCM quando admitidos no serviço de saúde já apresentam alterações relacionadas ao processo fibrótico. Venturini e colaboradores (2014) demonstraram que monócitos de pacientes com PCM-FC no momento do diagnóstico exibem alta produção de TGF-β1 e FGF. Adicionalmente, Tobón e colaboradores (2003), observaram que após a introdução do tratamento antifúngico o processo fibrótico é intensificado.

A forma crônica da PCM é mediada por uma resposta imunológica mista com predomínio dos perfis Th_{17} e Th_{22}, além da contribuição substancial do perfil Th_1. Essa resposta contribui parcialmente para a resistência a infecção, entretanto, está envolvida na exacerbação da resposta imunológica mediada por Th_{17} com consequente ativação de neutrófilos, lesão tecidual e desenvolvimento da fibrose(26–30).

Apesar dos mecanismos envolvidos no processo da fibrose serem pouco conhecidos, sugere-se que, a semelhança de outras doenças, a instalação da fibrose pulmonar na PCM é decorrente, também, da estimulação antigênica persistente e da constante ativação da resposta imune, além de distúrbios no processo de reparo tecidual(31). O processo crônico induz dano e morte tecidual e, assim, leva a produção de mediadores que ativam células vizinhas a produzir citocinas, quimiocinas e fatores de crescimento pró-fibróticos. Esses mediadores estimulam o recrutamento de leucócitos e a diferenciação de fibroblastos em

miofibroblastos, assim, aumentando a produção e deposição de colágeno. Além disso, a fibrogênese é caracterizada pela inibição das metaloproteinases de matriz extracelular (MMP)(32–35).

1.3. Drogas com potencial antifibrótico

1.3.1. Pentoxifilina. A pentoxifilina (PTX) é uma droga hemorreológica padrão, utilizada no tratamento de doenças vasculares crônicas(36). Estudos *in vitro* demonstraram que a atividade supressiva da droga sobre o TNF-α é similar aos efeitos do tratamento com anticorpo anti-TNF-α(37), podendo atuar também, na inibição da produção de IL-10(38,39). A droga possui potencial antifibrótico por exibir redução na proliferação de fibroblastos, supressão da produção, secreção e deposição de colágenos tipo I e III, proteoglicanas e fibronectina(40).

Apesar desses achados, os mecanismos envolvidos ainda não são totalmente conhecidos.

Experimentalmente foi testada em camundongos isogênicos da linhagem AKR/J infectados com *Schistosoma mansoni*. O tratamento diário com 200 mg/Kg de pentoxifilina, via injeção subcutânea na região dorsal, demonstrou que o perfil granulomatoso hepático dos animais tratados com a droga foi irregular e menos concêntrico, com redução significativa no tamanho das lesões nas fases aguda e crônica. O depósito de colágeno tipo I e III nas lesões foi menor nas duas fases da infecção e consequentemente houve redução da fibrose(41).

Desse modo, por exibir potencial antifibrótico, foi a primeira droga com essa característica a ser avaliada na PCM experimental murina. Neste estudo, a terapia combinada de ITC (1 mg/dia/animal) e PTX (20 mg/Kg) foi administrada diariamente via gavagem, em camundongos BALB/c machos que foram divididos em dois grupos: a) início do tratamento após 4 semanas de infecção; b) início do tratamento após 8 semanas de infecção. Após períodos de 4, 8, 12 e 16 semanas de tratamento, ambos os grupos resultaram em redução significativa da inflamação granulomatosa e da deposição de fibras colágenas nos pulmões, quando comparado a monoterapia com ITC(42).

Recentemente, o mesmo grupo demonstrou que 8 semanas de monoterapia com PTX (20mg/Kg/dia), dando início após 4 semanas de infecção (processo fibrótico precoce), foi capaz de reduzir a área de lesão dos pulmões associado a

redução da carga fúngica, além de restaurar os níveis de IFN-γ, CCL3 e IL-3(43).

Em um estudo realizado com dezoito pacientes com progressão da sarcoidose, tratados com 25 mg/Kg/dia da droga por seis meses, foi observado melhora clínica em onze pacientes. Sete permaneceram estáveis, não apresentaram piora clínica e apresentaram melhora na função pulmonar(44).

Em *clinical trial* realizado para o uso da PTX na cirrose avançada (ClinicalTrials.gov Identifier: NCT00162552), observou-se que a droga não reduziu o índice de mortalidade de pacientes com cirrose e insuficiência hepática, talvez por conta do curto período de tratamento de 6 meses. No entanto, esse tratamento oral com 400 mg da droga três vezes ao dia, limitou o risco de complicações relacionadas ao fígado, além de limitar o desenvolvimento de infecção bacteriana, insuficiência renal e encefalopatia hepática(45).

1.3.2. Talidomina. A talidomida (Thal) é uma droga com efeito anti-inflamatório e antineoplásico, que atua por inibir a produção de IL- 6 e TNF-α(46). Por apresentar, portanto, características similares a PTX, ela tem sido avaliada no tratamento da fibrose hepática em modelos experimentais(47–49). No modelo experimental de fibrose pulmonar induzida por bleomicina, o tratamento com Thal resultou em melhora da fibrose pulmonar com a diminuição na expressão de RNAm de IL-6, TGF-β1, VEGF, diminuição do depósito de colágeno tipo I e inibição da angiogênese(49). O efeito direto da Thal sobre fibroblastos e/ou degradação do colágeno, é desconhecido.

Até o momento foram realizados dois *clinical trials* para o tratamento de pacientes com fibrose pulmonar idiopática (ClinicalTrials.gov Identifier: NCT00600028 e NCT00162760). Embora tenham sido completados, apenas um deles teve seu resultado publicado. Nesse estudo, demonstrou-se que o tratamento oral com 400 mg/dia de Thal melhorou significativamente a pontuação do questionário sobre qualidade de vida *(Cough Quality of Life Questionnaire)* aplicado aos pacientes, em comparação aos indivíduos tratados com placebo, e, ainda, apresentou melhora significativa dos *scores* da tosse(50).

1.3.3. Azitromicina. A azitromicina (AZT), um importante antibiótico macrolídeo(51), tem demonstrado efeito antifibrótico promissor(52).

Em estudo experimental com cobaias que apresentavam inflamação

respiratória eosinofílica e foram tratadas com AZT, foi observado significativa redução dos reflexos da tosse, assim, os autores do estudo sugerem mesmo efeito em pacientes com fibrose pulmonar idiopática(53). Em modelo experimental murino de fibrose pulmonar induzida por bleomicina, o tratamento com AZT (3,5 mg/Kg/dia) resultou em redução do declínio da função pulmonar e redução da fibrose nos animais tratados. O estudo também demonstrou redução no recrutamento de linfócitos e neutrófilos, além de efeito imunomodulador diminuindo os níveis de IL-1β, IL-6, IL-17, MCP-1 e KC do fluido broncoalveolar(52).

Em estudo *in vitro*, os monócitos humanos desafiados com LPS e tratados com AZT apresentaram diminuição nos níveis de TNF-α e IL-12, em comparação aos monócitos apenas desafiados com LPS(54).

Em *clinical trial* (ClinicalTrials.gov Identifier: NCT00431964), realizado com pacientes pediátricos de fibrose cística sem coinfecção por *Pseudomonas aeruginosa*, foi observado que o tratamento com a droga em um período de 24 semanas, não afetou significativamente na melhora da função pulmonar(55).

Até o momento existe um *clinical trial* (ClinicalTrials.gov Identifier: NCT02173145) aberto para o recrutamento de pacientes com fibrose pulmonar idiopática.

2. Objetivo Geral

Avaliar o efeito antifibrótico das drogas PTX, AZT e Thal associados aos antifúngicos ITC e CMX, no tratamento da PCM pulmonar experimental.

2.1. Objetivos Específicos

2.1.1. Avaliar a influência do tratamento antifibrótico sobre a deposição de fibras colágenas e produção de hidroxiprolina nos pulmões.

2.1.2. Avaliar a influência do tratamento antifibrótico sobre a produção de TGF-β1, CCL3, IFN-γ, TNF-α, IL-10, VEGF, IL-6, IL-1β, IL-17 e IL-2 em homogenato dos pulmões.

2.1.3. Determinar a recuperação de fungos viáveis, peso corporal e a porcentagem das áreas com lesão pulmonar.

3. Referências bibliográficas

1. Teixeira MM, Theodoro RC, de Carvalho MJA, Fernandes L, Paes HC, Hahn RC, et al. Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. Mol Phylogenet Evol. agosto de 2009;52(2):273–83.

2. Lutz, A. Uma mycose pseudococcidica localizada na boca e observada no Brasil. Contribuição ao conhecimento das hyphoblastomycoses americanas. Bras Med.1908;(22):121-4;142-4

3. Muñoz JF, Farrer RA, Desjardins CA, Gallo JE, Sykes S, Sakthikumar S, et al. Genome Diversity, Recombination, and Virulence across the Major Lineages of Paracoccidioides. mSphere. outubro de 2016;1(5).

4. Matute DR, McEwen JG, Puccia R, Montes BA, San-Blas G, Bagagli E, et al. Cryptic speciation and recombination in the fungus Paracoccidioides brasiliensis as revealed by gene genealogies. Mol Biol Evol. janeiro de 2006;23(1):65–73.

5. Teixeira MM, Theodoro RC, Nino-Vega G, Bagagli E, Felipe MSS. Paracoccidioides species complex: ecology, phylogeny, sexual reproduction, and virulence. PLoS Pathog. outubro de 2014;10(10):e1004397.

6. Bagagli E, Franco M, Bosco SDMG, Hebeler-Barbosa F, Trinca LA, Montenegro MR. High frequency of Paracoccidioides brasiliensis infection in armadillos (Dasypus novemcinctus): an ecological study. Med Mycol. junho de 2003;41(3):217–23.

7. Naiff RD, Ferreira LC, Barrett TV, Naiff MF, Arias JR. [Enzootic paracoccidioidomycosis in armadillos (Dasypus novemcinctus) in the State of Pará]. Rev Inst Med Trop Sao Paulo. fevereiro de 1986;28(1):19–27.

8. Peraçoli MT, Sugizaki MF, Mendes RP, Naiff R, Montenegro MR. Paracoccidioides brasiliensis isolated from armadillos is virulent to Syrian hamsters. Mycopathologia. dezembro de 1999;148(3):123–30.

9. Bocca AL, Amaral AC, Teixeira MM, Sato PK, Sato P, Shikanai-Yasuda MA, et al. Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues. Future Microbiol. setembro de 2013;8(9):1177–91.

10. Moreto TC, Marques MEA, de Oliveira MLSC, Moris DV, de Carvalho LR, Mendes RP. Accuracy of routine diagnostic tests used in paracoccidioidomycosis patients at a university hospital. Trans R Soc Trop Med Hyg. agosto de 2011;105(8):473–8.

11. Mendes RP. The gamut of clinical manifestations. In: Franco M, Lacaz CS, Restrepo-Moreno A, Del Negro G, editors. Paracoccidioidomycosis. Boca Raton: CRC Press; 1994. p. 233-58

12. Jornal Brasileiro de Pneumologia - Google Livros [Internet]. [citado 11 de julho de 2017]. Disponível em: https://books.google.com.br/books?id=clDidh5b_icC&pg=PA170&lpg=PA170&dq=Tratamento+da+blastomicose+sul-americana+com+anfotericina+B+1958&source=bl&ots=psOVMdEb2k&sig=WiVhy1RQuIxCMIRbnAwe3Zt3aA4&hl=pt-BR&sa=X&ved=0ahUKEwju1unG3IHVAhWEjpAKHfJTDdUQ6AEIPjAD#v=onepage&q=Tratamento%20da%20blastomicose%20sul-americana%20com%20anfotericina%20B%201958&f=false

13. Barbosa W, Vasconcelos WM de P. AÇÃO DA SULFAMETOXAZOL ASSOCIADA AO TRIMETOPRIM NA TERAPÊUTICA DA BLASTOMICOSE SUL-AMERICANA. Rev Patol Trop. 1973;2(3).

14. Negroni R, Palmieri O, Koren F, Tiraboschi IN, Galimberti RL. Oral treatment of paracoccidioidomycosis and histoplasmosis with itraconazole in humans. Rev Infect Dis. fevereiro de 1987;9 Suppl 1:S47-50.

15. Restrepo A, Gomez I, Robledo J, Patiño MM, Cano LE. Itraconazole in the treatment of paracoccidioidomycosis: a preliminary report. Rev Infect Dis. fevereiro de 1987;9 Suppl 1:S51-56.

16. Naranjo MS, Trujillo M, Munera MI, Restrepo P, Gomez I, Restrepo A. Treatment of paracoccidioidomycosis with itraconazole. J Med Vet Mycol Bi-Mon Publ Int Soc Hum Anim Mycol. 1990;28(1):67–76.

17. Cavalcante R de S, Sylvestre TF, Levorato AD, de Carvalho LR, Mendes RP. Comparison between itraconazole and cotrimoxazole in the treatment of paracoccidiodomycosis. PLoS Negl Trop Dis. abril de 2014;8(4):e2793.

18. Campos EP, Cataneo AJ. [Pulmonary function in 35 patients with paracoccidioidomycosis]. Rev Inst Med Trop Sao Paulo. outubro de 1986;28(5):330–6.

19. Campos EP, Padovani CR, Cataneo AM. [Paracoccidioidomycosis: radiologic and pulmonary study in 58 cases]. Rev Inst Med Trop Sao Paulo. agosto de 1991;33(4):267–76.

20. Lemle A, Wanke B, Miranda JL, Kropf GL, Mandel MB, Mandel S. Pulmonary function in paracoccidioidomycosis (South American blastomycosis). An analysis of the obstructive defect. Chest. maio de 1983;83(5):827–8.

21. Santos WA dos, Silva BM da, Passos ED, Zandonade E, Falqueto A. Association between smoking and paracoccidioidomycosis: a case-control study in the State of Espírito Santo, Brazil. Cad Saúde Pública. fevereiro de 2003;19(1):245–53.

22. Tuder RM, el Ibrahim R, Godoy CE, De Brito T. Pathology of the human pulmonary paracoccidioidomycosis. Mycopathologia. dezembro de 1985;92(3):179–88.

23. Shikanai-Yasuda MA, Telles Filho F de Q, Mendes RP, Colombo AL, Moretti ML. [Guidelines in paracoccidioidomycosis]. Rev Soc Bras Med Trop. junho de 2006;39(3):297–310.

24. Cock AM, Cano LE, Vélez D, Aristizábal BH, Trujillo J, Restrepo A. Fibrotic sequelae in pulmonary paracoccidioidomycosis: histopathological aspects in BALB/c mice infected with viable and non-viable paracoccidioides brasiliensis propagules. Rev Inst Med Trop Sao Paulo. abril de 2000;42(2):59–66.

25. Araujo S de A. Contribuição ao estudo anátomo-clínico da Paracoccidioiodomicose em Minas Gerais. meio século de experiência - avaliação das necrópsias realizadas no período compreendido entre 1944 até 1999, no departamento de anatomia patológica e medicina legal, da Faculdade de Medicina da Universidade Federal de Minas Gerais. [Internet]. 2011 [citado 21 de junho de 2017]. Disponível em: http://www.bibliotecadigital.ufmg.br/dspace/handle/1843/BUOS-8KVLVK

26. de Castro LF, Ferreira MC, da Silva RM, Blotta MH de SL, Longhi LNA, Mamoni RL. Characterization of the immune response in human paracoccidioidomycosis. J Infect. novembro de 2013;67(5):470–85.

27. Benard G, Romano CC, Cacere CR, Juvenale M, Mendes-Giannini MJ, Duarte AJ. Imbalance of IL-2, IFN-gamma and IL-10 secretion in the immunosuppression associated with human paracoccidioidomycosis. Cytokine. 21 de fevereiro de 2001;13(4):248–52.

28. Mamoni RL, Nouér SA, Oliveira SJ, Musatti CC, Rossi CL, Camargo ZP, et al. Enhanced production of specific IgG4, IgE, IgA and TGF-β in sera from patients with the juvenile form of paracoccidioidomycosis. Med Mycol. 1º de janeiro de 2002;40(2):153–9.

29. Oliveira SJ, Mamoni RL, Musatti CC, Papaiordanou PMO, Blotta MHSL. Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls. Microbes Infect. 1º de fevereiro de 2002;4(2):139–44.

30. Mamoni RL, Blotta MHSL. Flow-cytometric analysis of cytokine production in human paracoccidioidomycosis. Cytokine. 1º de agosto de 2006;35(3):207–16.

31. Kisseleva T, Brenner DA. Fibrogenesis of parenchymal organs. Proc Am Thorac Soc. 15 de abril de 2008;5(3):338–42.

32. Phan SH. Biology of fibroblasts and myofibroblasts. Proc Am Thorac Soc. 15 de abril de 2008;5(3):334–7.

33. Klingberg F, Hinz B, White ES. The myofibroblast matrix: implications for tissue repair and fibrosis. J Pathol. janeiro de 2013;229(2):298–309.

34. Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmoulière A, Varga J, et al. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. Am J Pathol. abril de 2012;180(4):1340–55.

35. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. janeiro de 2008;214(2):199–210.

36. Jull A, Arroll B, Parag V, Waters J. Pentoxifylline for treating venous leg ulcers. Cochrane Database Syst Rev. 18 de julho de 2007;(3):CD001733.

37. Tilg H, Eibl B, Pichl M, Gächter A, Herold M, Brankova J, et al. Immune response modulation by pentoxifylline in vitro. Transplantation. julho de 1993;56(1):196–201.

38. Marcinkiewicz J, Grabowska A, Lauterbach R, Bobek M. Differential effects of pentoxifylline, a non-specific phosphodiesterase inhibitor, on the production of IL-10, IL-12 p40 and p35 subunits by murine peritoneal macrophages. Immunopharmacology. setembro de 2000;49(3):335–43.

39. Bienvenu J, Doche C, Gutowski MC, Lenoble M, Lepape A, Perdrix JP. Production of proinflammatory cytokines and cytokines involved in the TH1/TH2 balance is modulated by pentoxifylline. J Cardiovasc Pharmacol. 1995;25 Suppl 2:S80-84.

40. Samlaska CP, Winfield EA. Pentoxifylline. J Am Acad Dermatol. abril de 1994;30(4):603–21.

41. Mati VLT, Freitas RMC, Melo AL. Effects of pentoxifylline during Schistosoma mansoni infection in Swiss mice: an analysis of worm burden, fecundity and liver histopathology. J Helminthol. dezembro de 2010;84(4):348–54.

42. Naranjo TW, Lopera DE, Diaz-Granados LR, Duque JJ, Restrepo AM, Cano LE. Combined itraconazole-pentoxifylline treatment promptly reduces lung fibrosis induced by chronic pulmonary paracoccidioidomycosis in mice. Pulm Pharmacol Ther. fevereiro de 2011;24(1):81–91.

43. Lopera DE, Naranjo TW, Hidalgo JM, Echeverri L, Patiño JH, Moreno ÁR, et al. Pentoxifylline immunomodulation in the treatment of experimental chronic pulmonary paracoccidioidomycosis. Fibrogenesis Tissue Repair. 1º de junho de 2015;8:10.

44. Zabel P, Entzian P, Dalhoff K, Schlaak M. Pentoxifylline in treatment of sarcoidosis. Am J Respir Crit Care Med. maio de 1997;155(5):1665–9.

45. Lebrec D, Thabut D, Oberti F, Perarnau J-M, Condat B, Barraud H, et al. Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis. Gastroenterology. maio de 2010;138(5):1755–62.

46. Zhou S, Wang F, Hsieh T-C, Wu JM, Wu E. Thalidomide-a notorious sedative to a wonder anticancer drug. Curr Med Chem. 2013;20(33):4102–8.

47. Arai H, Furusu A, Nishino T, Obata Y, Nakazawa Y, Nakazawa M, et al. Thalidomide prevents the progression of peritoneal fibrosis in mice. Acta Histochem Cytochem. 28 de abril de 2011;44(2):51–60.

48. Choe J-Y, Jung H-J, Park K-Y, Kum Y-S, Song GG, Hyun D-S, et al. Anti-fibrotic effect of thalidomide through inhibiting TGF-beta-induced ERK1/2 pathways in bleomycin-induced lung fibrosis in mice. Inflamm Res Off J Eur Histamine Res Soc Al. março de 2010;59(3):177–88.

49. Tabata C, Tabata R, Kadokawa Y, Hisamori S, Takahashi M, Mishima M, et al. Thalidomide prevents bleomycin-induced pulmonary fibrosis in mice. J Immunol Baltim Md 1950. 1º de julho de 2007;179(1):708–14.

50. Horton MR, Santopietro V, Mathew L, Horton KM, Polito AJ, Liu MC, et al. Thalidomide for the treatment of cough in idiopathic pulmonary fibrosis: a randomized trial. Ann Intern Med. 18 de setembro de 2012;157(6):398–406.

51. Retsema J, Girard A, Schelkly W, Manousos M, Anderson M, Bright G, et al. Spectrum and mode of action of azithromycin (CP-62,993), a new 15-membered-ring macrolide with improved potency against gram-negative organisms. Antimicrob Agents Chemother. dezembro de 1987;31(12):1939–47.

52. Wuyts WA, Willems S, Vos R, Vanaudenaerde BM, De Vleeschauwer SI, Rinaldi M, et al. Azithromycin reduces pulmonary fibrosis in a bleomycin mouse model. Exp Lung Res. dezembro de 2010;36(10):602–14.

53. Tokuda A, Ohkura N, Fujimura M, Furusho S, Abo M, Katayama N. Effects of macrolides on antigen-induced increases in cough reflex sensitivity in guinea pigs. Pulm Pharmacol Ther. fevereiro de 2010;23(1):55–61.

54. Khan AA, Slifer TR, Araujo FG, Remington JS. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. Int J Antimicrob Agents. fevereiro de 1999;11(2):121–32.

55. Saiman L, Anstead M, Mayer-Hamblett N, Lands LC, Kloster M, Hocevar-Trnka J, et al. Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with Pseudomonas aeruginosa: a randomized controlled trial. JAMA. 5 de maio de 2010;303(17):1707–15.

Manuscrito

Evaluation of antifibrotic and antifungal combined therapies in experimental pulmonary paracoccidioidomycosis

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Running title: Antifibrotic therapies in paracoccidioidomycosis

Keywords: *Paracoccidioides brasiliensis*; pulmonary fibrosis; itraconazole; cotrimoxazole; pentoxifylline; azithromycin; thalidomide

Abbreviations:

AZT	Azithromycin
CF	Chronic form
CFU	Colony forming units
CMX	Cotrimoxazole
H/E	Hematoxylin and eosin
ITC	Itraconazole
OH-proline	Hydroxyproline
Pb	*Paracoccidioides brasiliensis*
PBS	Phosphate buffered saline
PCM	Paracoccidioidomycosis
PF	Pulmonary fibrosis
PTX	Pentoxifylline
Thal	Thalidomide

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ABSTRACT

Objetives - To evaluate three different antifibrotics therapies (pentoxifylline-PTX, azithromycin-AZT and thalidomide-Thal) associated with itraconazole (ITC) or cotrimoxazole (CMX) in a murine model of pulmonary paracoccidioidomycosis.

Methods - BALB/c mice were inoculated with *Paracoccidioides brasiliensis* (Pb) and after 8 weeks, were submitted to different treatments as following: PTX/ITC, PTX/CMX, AZT/ITC, AZT/CMX, Thal/ITC and Thal/CMX. After 8 weeks of follow-up, the lung were collected for determination of fungal burden, production of OH-proline, deposition of reticulin fibers, and levels of cytokines and growth factors.

Results - Pb-infected mice treated with PTX/ITC showed reduction in the levels of OH-proline associated with lower production of IL-6, IL-17 and TGF-β1 and higher levels of IL-10. Similarly, Pb-infected mice treated with AZT/CMX exhibited less pulmonary fibrosis associated with decreased levels of TGF-β1 and increased TNF-α and IL-10. Both groups that were treated with ITC/Thal and CMX/Thal showed intense weight loss, increased deposition of reticulin fibers, high levels of CCL3, IFN-γ and VEGF, and decreased levels of IL-6, IL-1β, IL-17 and TGF-β1.

Conclusions - Our findings reinforce the anti-fibrotic role of PTX in pulmonary PCM, but only when associated with ITC. Besides, our results point out the AZT as a candidate for antifibrotic drug in association with CMX.

BACKGROUND

Paracoccidioidomycosis (PCM) is a systemic granulomatous mycosis caused by fungi of *Paracoccidioides* genus that is restrict to Latin America, from Mexico to Argentina.[1] It is estimated an incidence of 1-3 cases per 100,000 habitants and the most affect individuals are male rural workers. [2]

There are three main clinical forms: i)acute/subacute form that, in general, compromises children, teenagers and young adults and the clinical manifestations involve organs rich in mononuclear phagocytic cells; ii) chronic form (CF) that commonly affects adult males from 30 years-old and usually compromises lungs and mucosa of the upper aerodigestive tract; iii) residual form that is characterized by sequelea, especially in lungs, larynx and andrenals[3]. Most of patients show pulmonary fibrosis (PF) since their first admission and usually become worst during the treatment. [4–6] Although fibrogenesis is recognized as an early process,[4,7–9] the mechanisms involved in the PCM-PF process are poorly understood. It is suggested that the establishment of pulmonary fibrosis in PCM is due to persistent fungal antigen stimulation and constant activation of the immune response, as well as disturbances in the tissue repair mechanisms.

The treatment of PCM is based on antifungal drugs such as amphotericin B, sulfamethoxazole-trimethoprim (also called cotrimoxazole - CMX), and imidazolic compounds, such as itraconazole (ITC) that has been considered the first choice for the treatment of PCM. Although the antifungal therapies are considered safe and efficacious, the treatment is longer and the treatment of PF is only symptomatic.

The association of pentoxifylline (PTX) and ITC was the first combined therapy successfully evaluated in a murine model of chronic experimental PCM.[10] The anti-

fibrotic effect of PTX/ITC was characterized by reduction in the granulomatous inflammation and fibrosis in the lungs. Several studies have been performed using PTX to diminish PF and results demonstrate non-progressing disease.[11,12] PTX improves the rheological properties of blood, exhibits anti-inflammatory properties and antioxidative acitivity.[13] Recently, the effectiveness of PTX in the treatment of fibrosis has attenuated and reversed[13] by reduces the production of TNF, IL-1α, IL-6, and IL-8,[14] and therefore to prevent adherence, migration and degranulation of leukocytes.[15] Additionally, PTX shows antiproliferative effect on fibroblasts and inhibits extracellular matrix synthesis.[16,17]

Azithromycin (AZT) is a macrolide antibiotic that has been also reported as candidate as therapy in PF[18,19] since their immunomodulatory effects have been demonstrated in experimental bleomycin-induced fibrosis by decreasing production of IL-1β, IL-6, IL-17, MCP-1 and KC. *In vitro* studies have shown less production of TNF-a and IL-12 by human monocytes and IL-8 by human smooth muscle cells.[20–22]

Thalidomide (Thal) is longer recognized as potent inhibitor of TNF-α and IL-6.[23] Considering its role in chronic inflammation, it has been evaluated in experimental model of bleomycin-pulmonary fibrosis[24,25] and in idiopathic pulmonary fibrosis.[26] The results of these studies have shown decreased deposition of type I collagen in the lungs.[25]

Considering that new easily access options for PF treatment and the absence of studies evaluating antifibrotic therapies and CMX, a drug that is largely used and free distributed in Brazil; in the present study, we investigated the influence of PTX, AZT and Thal associated with ITC or CMX in a murine model of pulmonary PCM.

METHODS

Fungus

P. brasiliensis (isolate 326), recently isolated from a patient in Botucatu, SP, Brazil, was maintained by incubation at 35ºC in the yeast phase, with biweekly subcultures, in GPY culture medium (2% glucose, 1% peptone and 0.5% yeast extract). The viability of the fungus was monitored by staining with blue cotton lactophenol.

Mice and experimental infection

Isogenic BALB/c male mice, 8-12 weeks old were obtained from Lauro de Souza Lima Institute, Bauru, SP, Brazil. The mice were kept under adequate environmental conditions, receiving balanced feed and water *ad-libitum*. Mice were inoculated with 10^6 *P. brasiliensis* yeast forms by intratracheal route in phosphate buffered saline (PBS), while uninfected animals received only PBS. For the intratracheal inoculation, mice were anesthetized with the intraperitoneal administration of ketamine (80mg/Kg) and xylazine (10 mg/Kg).

Drug treatments

All treatments were performed as single dose by gavage in the morning. CMX (Bactrim®, Roché, Brazil) was daily administered at concentration of 200mg/Kg of body weight.[27,28] ITC (Sempera®, Jansen, Germany) was daily administered at concentration of 1mg/mouse.[10] Thal (FundaçãoEzequiel Dias, Brazil) was administered at concentration of 40mg/mL prepared in carboxymethylcellulose (Sigma-Aldrich, Germany) in 5 days/week.[25,29] PTX (Sigma-Aldrich) was daily administered at concentration of 20mg/Kg of body weight.[10] AZT (Pfizer, Belgium)

was administered day-on day-off at a concentration of 3.5mg/Kg of body weight [21].

Experimental design

Mice were randomly distributed in groups according to four combined therapies: 1) antifibrotic and antifungal combined treatment; 2) antifibrotic and placebo combined treatment; 3) antifungal and placebo combined treatment; 4) placebo treatment. All treatments were initiated 8 weeks after infection. Detalied information about the infection on the week 8 is shown in the Figure 1. After 8 weeks of treatment (16 weeks p.i.), mice were euthanized. Mice were weighed weekly since first week of treatment until the 8th week and it was calculated the percentage of gain or loss weight. All procedures were performed according to the ethical standards established by Brazilian College of Animal Experimentation and the project was approved (015/2016-CEUA-FC) by Animal Experimentation Ethics Committee of the Faculdade de Ciências, Unesp, Bauru, SP, Brazil.

Collection of biological material

Mice were anesthetized with inhalation of isoflurane followed by inhalation of carbon dioxide. Autopsy was performed to collect the lungs according to Supplementary Figure 1.

Histopathological analysis

Fragments of lungs were removed and fixed in 10% buffered formalin. Paraffin-embedded sections (4µm) were stained with hematoxylin and eosin (H/E) to evaluation of the inflammation, and Gomor's reticulin for deposition of collagen III fibers. The images were captured in digital camera attached to the optical

microscope (Axiostar HBO plus 50/AC Fluorescence Microscopy, Carl Zeiss, Germany) and analyzed in Image J software (version 1.51k, National Institutes of Health, USA).

Recovery viable fungi

Samples of lungs were weighed and macerated in 1.0 ml of sterile PBS and a volume of 100 µl was spread over culture plates, using a Drigalski T loop, containing BHI medium supplemented with 4% horse serum and 1% gentamycin. The plates were sealed and incubated at 35°C for 1-2 weeks. The number of colony forming units (CFU) was normalized per gram of tissue and $\log_{10}$ transformed.

Dosages of growth factors and cytokines

Levels of TGF-β1, CCL3, IFN-γ, TNF-α, IL-10, VEGF, IL-6, IL-1β, IL-17 and IL-2 in the lung homogenates were measured using a cytokine Duo-Set Kit (R&D Systems, Minneapolis, MI, USA), according to the manufacturer's instructions.

Hydroxyproline assay

Hydroxyproline (OH-proline) is an amino acid and a major building block of collagen. An increase in total OH-proline content in tissue samples is an indicator of the increased deposition of collagen fibers.[30] Briefly, samples were hydrolyzed with 6 N HCl at 110°C for 16 hours, then filtered and mixed with methanol and evaporated by a vacuum concentrator. The crystallized samples were dissolved in 50% isopropanol and incubated with 0.6% chloramine-T for 10 minutes. A volume of 100µL Ehrlich's reagent was added, and samples were incubated at 50°C for 45 minutes under constant shaking. Samples were measured at 540 nm, and concentrations of total

lung OH-proline were calculated against a standard curve.

Statistical analyzes

All statistical tests were performed using GraphPadInStat software version 3.0 for Windows (GraphPad Software, San Diego, CA, USA) and the significance level established to reject the 5% null hypothesis. ANOVA test with Tukey post-test.

RESULTS

PTX/ITC combined therapy decreased the deposition of collagen by down-regulates the inflammation in the lungs. In the present study, we initiated the antifibrotic and antifungal combined therapies in the 8th week of infection, i.e., in a consolidated stage of tissue granulomatous response and well-defined fibrotic process that mimic the situation observed in patients during their admission/diagnosis.[7,9] Pb-infected mice before treatment showed pulmonary lesion in all lobes corresponding to 65% of tissue area (Fig. 1B). The lesions were characterized by large and confluent granulomas composed of yeast cells, epithelioid cells and giant cells in the center surrounded by lymphocytes and mononuclear cells in the periphery. In addition, it was observed several focus with inflammatory infiltrate of polymorphonuclear cells and few areas of necrosis (Fig. 1B). Deposition of reticulin fibers was intense in the periphery of the granulomas, bronchi and arteries (Fig. 1C). We also observed high recovery of viable fungi (Fig. 1A) and the Pb-infected mice exhibited higher levels of IL-6, IL-1β, CCL3, IL-10, TGF-β, VEGF and IFN-γ (Fig. 1D - J) in the lungs than non-infected mice..

After 8 weeks of PTX/ITC combined therapy, Pb-infected mice exhibited a decreased production of OH-proline and gain of body weight compared to non-treated Pb-infected mice and ITC-treated Pb-infected mice, respectively (Figs. 2B, 2C). In contrast, Pb-infected mice treated with PTX/CMX showed increased deposition of reticulin fibers compared to non-treated Pb-infected mice (Fig. 2B). In addition, PTX/CMX and CMX-treated Pb-infected mice showed loss body weight in comparison to non-treated Pb-infected mice (Fig. 2C). As expected, the therapies did not interfere in lesion area and fungal burden (data not shown) since 8 weeks of

treatment is considered short and we opted to evaluated the antifibrotic properties of the drugs during active disease and not during remising phase to avoid misunderstanding.

In order to underly the possible mechanisms involved and understand the differences of PTX responses observed in association with ITC and CMX, we determined the levels of cytokines and growth factors in the pulmonary milieu. First, we characterized the effect of ITC and CMX treatments in the local immune response of Pb-infected. While ITC-treated Pb-infected mice showed overproduction of IL-1β (Fig. 3B), TNF-α (Fig. 3C) and IL-10 (Fig. 3F) than non-treated Pb-infected mice; CMX-treated Pb-infected mice showed similar production of pro-inflammatory cytokines and less production of IL-17 (Fig. 3E) and TGF-β1 (Fig. 3H). PTX/ITC combined therapy induced lower levels of IL-1β (Fig. 3B) and TGF-β1 (Fig. 3H) than ITC-treated Pb-infected mice. PTX/CMX combined therapy showed similarity with CMX-treated Pb-infected mice, except for tendency toward high production of CCL3 (Fig. 3D) and IL-1β (Fig. 3B).

AZT/CMX combined therapy decreased deposition of collagen by induces a pro-inflammatory response regulated by IL-10. AZT/CMX combined therapy decreased the production of OH-proline (Fig. 4A) and restored the loss body weight in comparison to CMX-treated Pb-infected mice (Fig. 4C). We observed that AZT/CMX triggered a tendency toward higher production of IL-17 (Fig. 5G) and IL-10 (Fig. 5H) in Pb-infected mice than in CMX-treated Pb-infected mice.

On the other hand, the AZT/ITC combined therapy induced tendency toward higher deposition of reticulin fibers ($p=0.06$) and significant higher loss weigh body in Pb-infected mice than ITC-treated and non-treated Pb-infected mice (Figs. 4B, 4C).

Although AZT/ITC combined therapy induced low production of IL-6 (Fig. 5B), IL-1β (Fig. 5C), IL-10 (Fig. 5H), IL-2 (Fig. 5I) and TGF-β1 (Fig. 5J) compared to ITC-treated Pb-infected mice, we observed higher levels of VEGF (Fig. 5A), IFN-y (Fig. 5D), CCL3 (Fig. 5F), and IL-17 (Fig. 5G) than ITC-treated or non-treated Pb-infected mice.

Thal/ITC and Thal/CMX combined therapies increased pulmonary fibrosis and severe disease was also observed. Thal/ITC and Thal/CMX combined therapies exhibited similar results. Both treatments increased the deposition of reticulin fibers (Fig. 6B) compared to non-treated and antifungal-treated Pb-infected mice, following a marked loss weight body (Fig. 6C). As expected, Thal triggered lower production of IL-6 (Fig. 7B), IL-1β (Fig. 7C), IL-17 (Fig. 7F) and TGF-β1 (Fig. 7I). On the other hand, the both combined therapy induced high levels of VEGF (Fig. 7A), IFN-y (Fig. 7D) and CCL3 (Fig. 7E). Additionally, levels of IL-10 (Fig. 7G) and IL-2 (Fig. 7H) were decreased in Thal/ITC-treated Pb-infected mice.

Almost half of mice submitted to treatment with Thal/CMX was euthanized at the 6th week because they exhibited neurological alterations characterized by loss of tonicity of the tail, hind legs, and changes in balance, resulting in the inclination to one side and consequently circular movements when walking and when held by the tail. Histopathological analysis of the brain showed yeast cells compatible to *P. brasiliensis* and discrete inflammatory infiltrate (Suppl. Fig. 3 and 4).

DISCUSSION

The present model of pulmonary PCM showed similar pattern of pulmonary lesions and fibrosis as observed in the murine model of PCM induced by conidia of Pb as well as in patients.[4,28,31,32] High levels of all immunological parameters evaluated in the present study reinforce several features of PCM patients with chronic form: intense pro-inflammatory response, Th1/Th17 profile and high production of mediators involved in fibrogenesis. [33–37]

In general, our findings showed that the antifibrotic drugs did not show the same effect when associated with ITC and CMX. While PTX showed better anti-fibrotic effect in combination with ITC, as previously reported,[10] the same effect was not observed in combination with CMX. Similarly, AZT showed anti-fibrotic property in combination with CMX, but not with ITC. Recently, Cavalvante *et al* [38] have showed relevant differences between ITC and CMX in the treatment of PCM-patients, such as faster clinical cure in chronic patients treated with ITC. Indeed, we believe that the one important difference observed in the combined therapies should be associated with the fungal death rate. It is well known that ITC present more efficient antifungal effect than CMX.[38,39] In experimental studies, the differences is also more pronounced. In the model of pulmonary PCM inoculated with conidia of *P. brasilienis*, the fungal clearance is observed from the 8th week of treatment with ITC started in the 4th week of the inoculation.[10] Using the same model of the present study, we observed fungal clearance after 20 weeks of treatment with CMX started in the 4th week of the inoculation (Venturini *et al*, in submission). For ethical reasons, in our protocols, CMX was administered in single dose per day instead twice (while the half-life of CMX is 12 hours).[28] Therefore it is possible that the anti-inflammatory effect of

PTX impaired a regulated immune response since increased levels of IL-1β, CCL3, TNF-α was observed in PTX/CMX-treated Pb-infected mice. Several studies have demonstrated that intense inflammation is an important factor in fibrogenesis.[31] Considering the limitation of our study and the decreased levels of TGF-β1 observed in the lungs of these mice, we do not exclude the possible antifibrotic role of PTX in association with CMX. Nevertheless it is important to highlight that the compliance of patients during the treatment using CMX is essential to the success of antifibrotic treatment.

In the present study, we observed that the immunological profile of CMX-treated Pb-infected mice was similar to the non-treated Pb-infected mice. ITC-treated Pb-infected mice showed overproduction of IL-1β and TNF-α and, as compensatory mechanism, high production of IL-10. The success of AZT as anti-fibrotic seems to be dependent of inflammatory milieu of the host. In the microenvironment with exacerbated pro-inflammatory response, such as the AZT/ITC combined therapy, although the reduction of inflammation was observed, it was not enough to drive an antifibrotic effect. In AZT/CMX-treated Pb-infected mice, the inflammatory status induced by infection associated with lower levels of TGF-β1 and IL-17 and high levels of IL-10, propitiated an anti-fibrotic effect. Murine model of induced-bleomycin pulmonary fibrosis was observed that treatment with AZT was able to decrease production of IL-1β, IL-6 and IL-17, increase IL-10 and IL-2, besides decrease MCP-1 and KC led to decreasing migration of macrophages and neutrophils, resulting in improvement of pulmonary fibrosis.[21] Other authors suggest that the positive effects of AZT on fibrosis are related to a decrease in neutrophil recruitment.[20,22,40] Our findings reinforce that an effective antifibrotic drug is not only associated with down-regulation of mediators directly involved in the activation and/or differentiation of

fibroblasts, such as TGF-β1, but also in promote a regulated immune response. As above mentioned, PCM-patients usually present intense inflammatory response; however, the immune profile of these patients after the introduction of ITC or CMX drugs remains to be determined. Therefore, our findings reinforce the need to researches evaluating the immune profile of PCM-patients under treatment.

Although Thal has been used in experimental studies in fibrosis and clinical studies are ongoing, they have been performed under conditions where the etiology of the disease is not infectious.[24–26,41] In the present study, the anti-inflammatory activity of the drug was shown to be pronounced, however, impairing the action of antifungal, triggering the worsening of the disease, such as the spread to central nervous system (CNS) and accompanied by an increase in pulmonary fibrosis. Although the peripheral neuropathies is a common side effect of Thal in treatment, as observed in patients with cancer,[42–44] we suspected the presence of fungi in the CNS because non-infected mice treated with Thal did not exhibit these signals. Indeed, our histopathological findings and clinical signals were similar to a murine model of neuroparacoccidiodomycosis in with the fungus is directly inoculated in the brain [45].

The use of Thal induced more PF instead its control and it was marked by increased levels of CCL3 and VEGF. Interestedly, the increased levels of these mediators were also observed in Pb-infected mice submitted to combined therapies that failed to control the PF (PTX/CMX and AZT/ITC). High levels of CCL3 have been previously associated with worsening of fibrosis. It was observed that knockout mice for the *CCL3* gene showed a significant reduction of fibrosis, this same study demonstrated *in vitro* that CCL3 led to increased proliferation and migration of collagen producing cells [46]. VEGF gene silencing and its signaling pathway blockade are capable of attenuating pulmonary fibrosis[47,48].

In conclusion, our findings reinforce the antifibrotic role of pentoxifylline in pulmonary PCM, associated with itraconazole. The azithromycin associated with cotrimoxazole showed antifibrotic effect and is a candidate to antifibrotic drug in treatment of PCM. The antifibrotic mechanisms involved were related to decrease production of TGF-β1 and increased IL-10. Treatment with thalidomide was not satisfactory in any associations. Intensification of pulmonary fibrosis in Pb-infected mice was associated with non-regulated inflammation and also with elevated levels of CCL3 and VEGF in the lungs. Thus, the results obtained open new perspectives for the therapeutic application of the drugs evaluated in PCM, improving the quality of life of patients.

Acknowledgements

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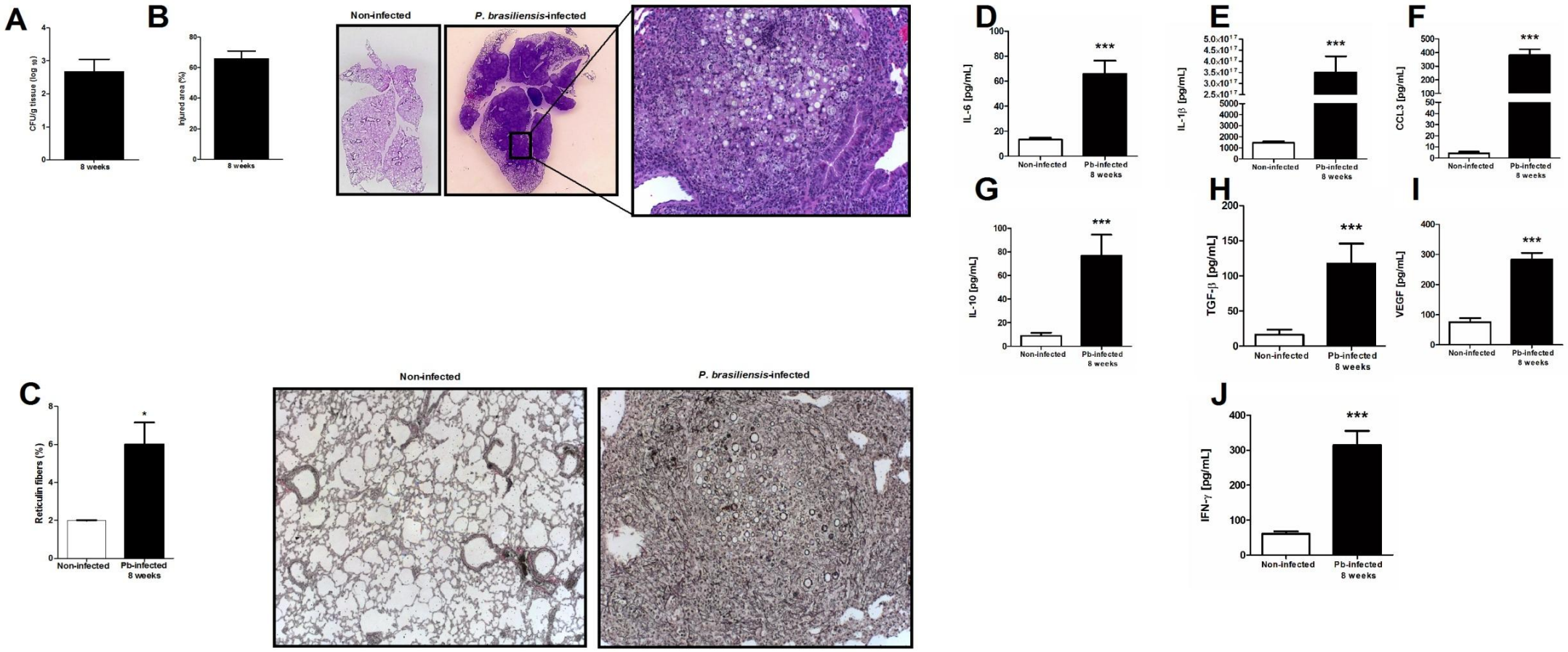

Figure 1. Characterization of pulmonary infection of male BALB/c mice by intratracheal inoculation of *P. brasiliensis* isolated 326 at 8 weeks p.i. A) Recovery of viable lung homogenates fungi. Data expressed in $\log_{10}$. B) Determination of the area of lung tissue injury. Image of healthy tissue without lesions followed of an 8-weeks p.i. lung, is possible to observe an extensive lesion area characterized by the presence of granulomas (H/E). C) Determination of the percentage of reticulin fibers in the lungs. Inflammatory mediators of pulmonary homogenate: D) IL-6, E) IL-1β, F) CCL3, G) IL-10, H) TGF-β, I) VEGF, J) IFN-γ. Unpaired T test; **p* <0.05; ***p* <0.01; ****p* <0.001, n=5-7.

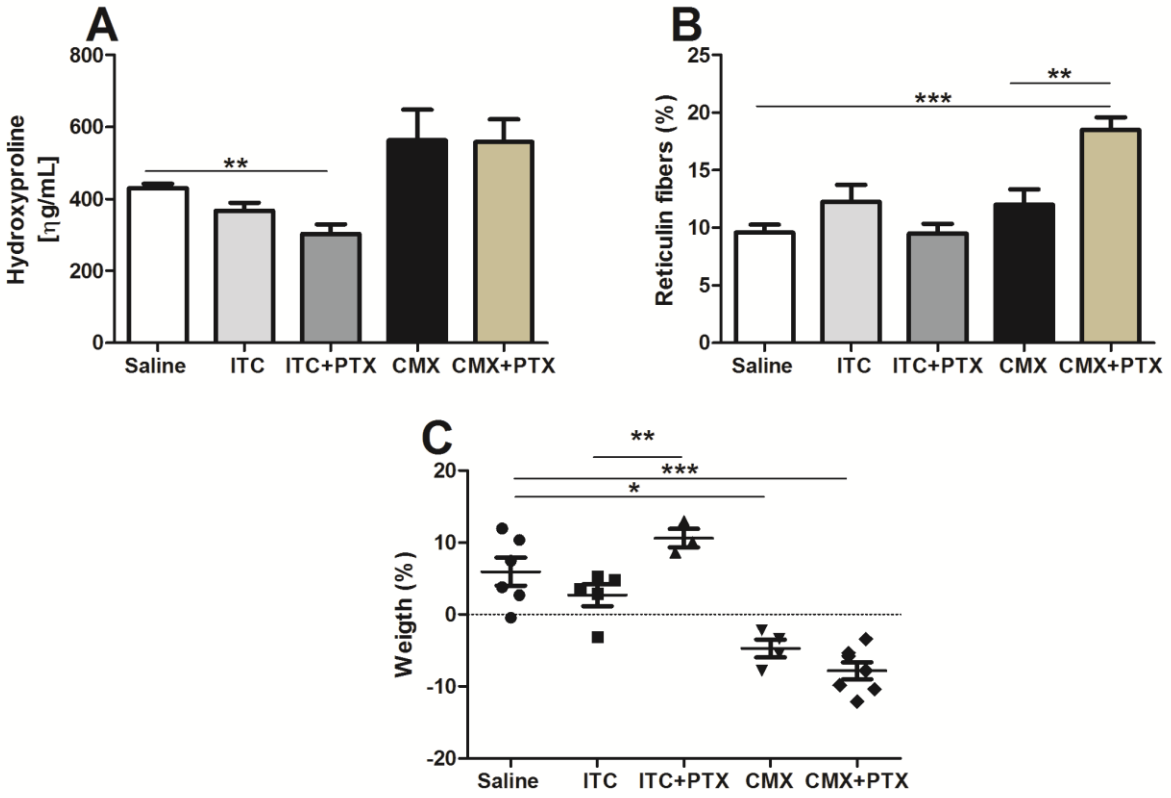

Figure 2. Evaluation of pentoxifylline (PTX) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) on clinical, histopathological and pulmonary fibrosis parameters. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the following therapeutic regimens were started: PTX/ITC, PTX/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) Quantification of hydroxyproline in the lungs. Values expressed in ηg/mL. B) Determination of the percentage of reticulin fibers in the lungs. C) Weight variation between the beginning and the end of the treatment. Data expressed as a percentage (%). ANOVA test with Tukey post-test; *p <0.05; **p <0.01; ***p <0.001, n=5-7.

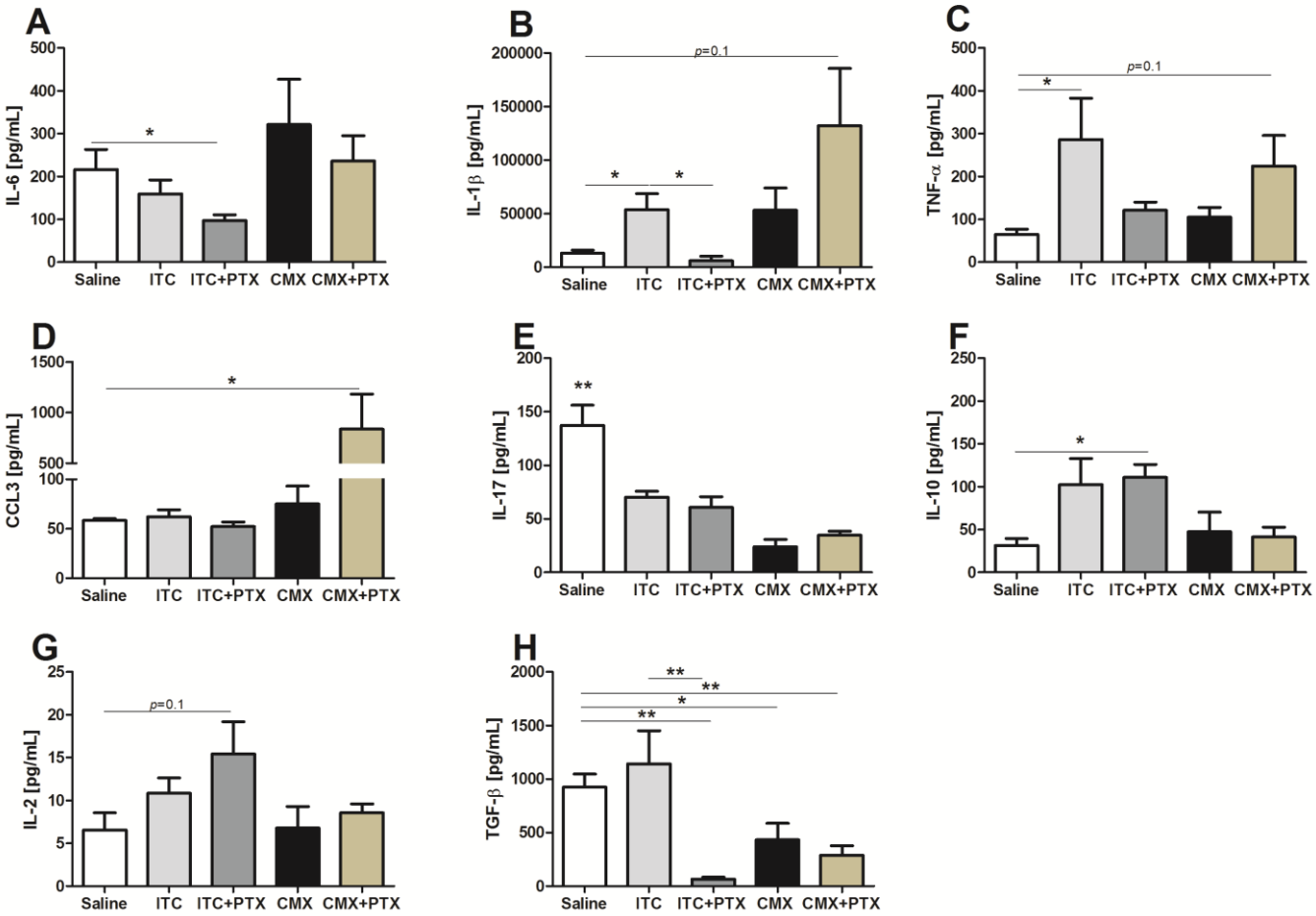

Figure 3. Influence of pentoxifylline (PTX) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) in the quantification of inflammatory mediators and growth factors in the lungs. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the following therapeutic regimens were started: PTX/ITC, PTX/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) IL-6, B) IL-1β, C) TNF-α, D) CCL3, E) IL-17, F) IL-10, G) IL-2 and H) TGF-β. ANOVA test with Tukey post-test; **p* <0.05; ***p* <0.01; ****p* <0.001, n=5-7.

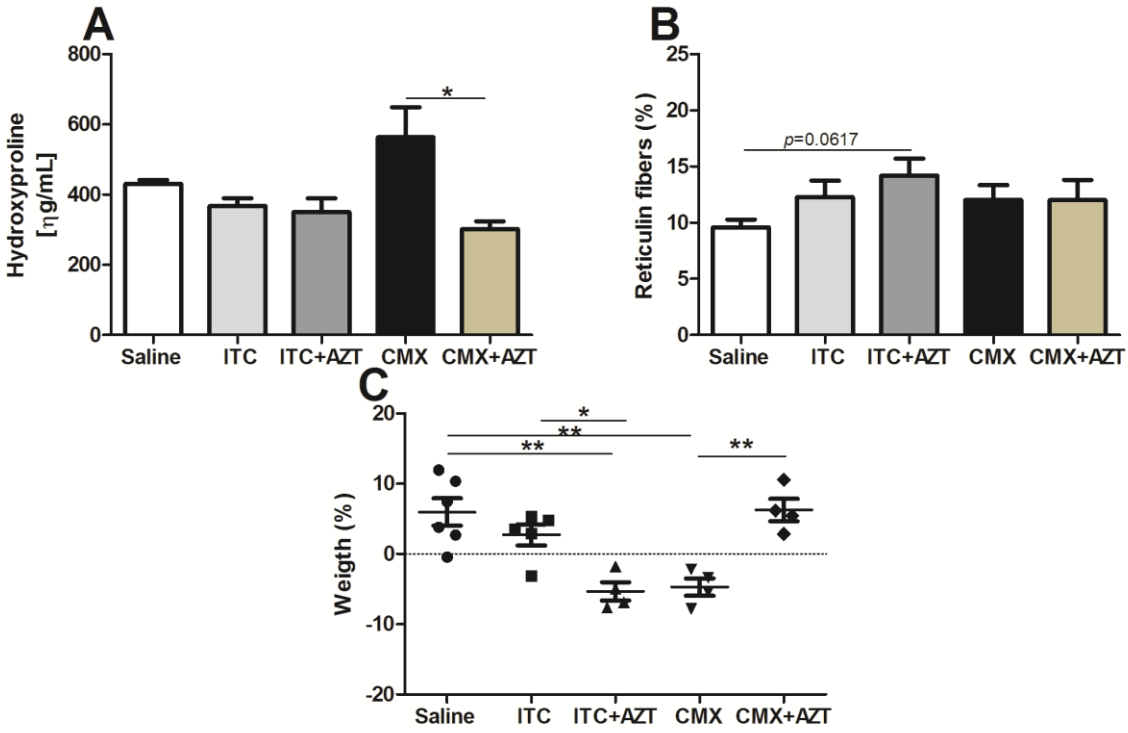

Figure 4. Evaluation of azithromycin (AZT) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) on clinical, histopathological and pulmonary fibrosis parameters. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the following therapeutic regimens were started: AZT/ITC, AZT/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) Quantification of hydroxyproline in the lungs. Values expressed in ηg/mL. B) Determination of the percentage of reticulin fibers in the lungs. C) Weight variation between the beginning and the end of the treatment. ANOVA test with Tukey post-test; **p* <0.05; ***p* <0.01; ****p* <0.001, n=5-7.

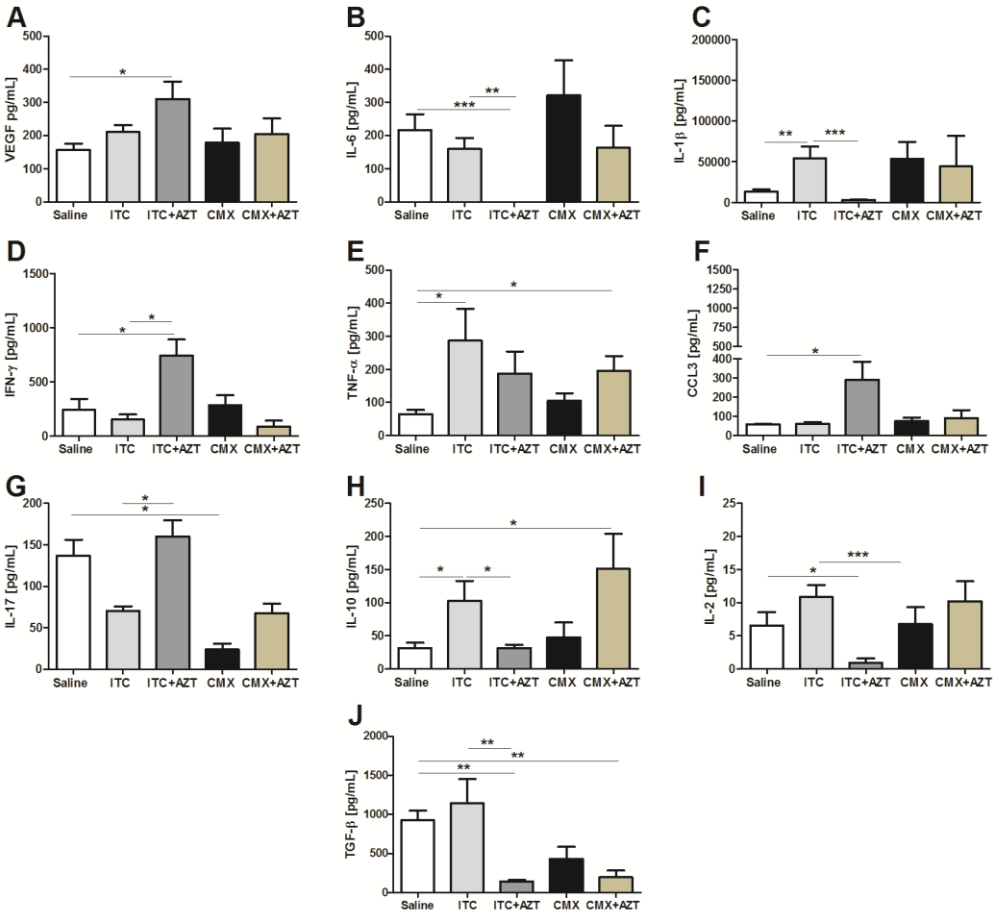

Figure 5. Influence of azithromycin (AZT) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) in the quantification of inflammatory mediators and growth factors in the lungs. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the following therapeutic regimens were started: AZT/ITC, AZT/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) VEGF, B) IL-6, C) IL-1β, D) IFN-γ, E) TNF-α, F) CCL3, G) IL-17, H) IL-10, I) IL-2 and K) TGF-β. ANOVA test with Tukey post-test; *p <0.05; **p <0.01; ***p <0.001, n=5-.
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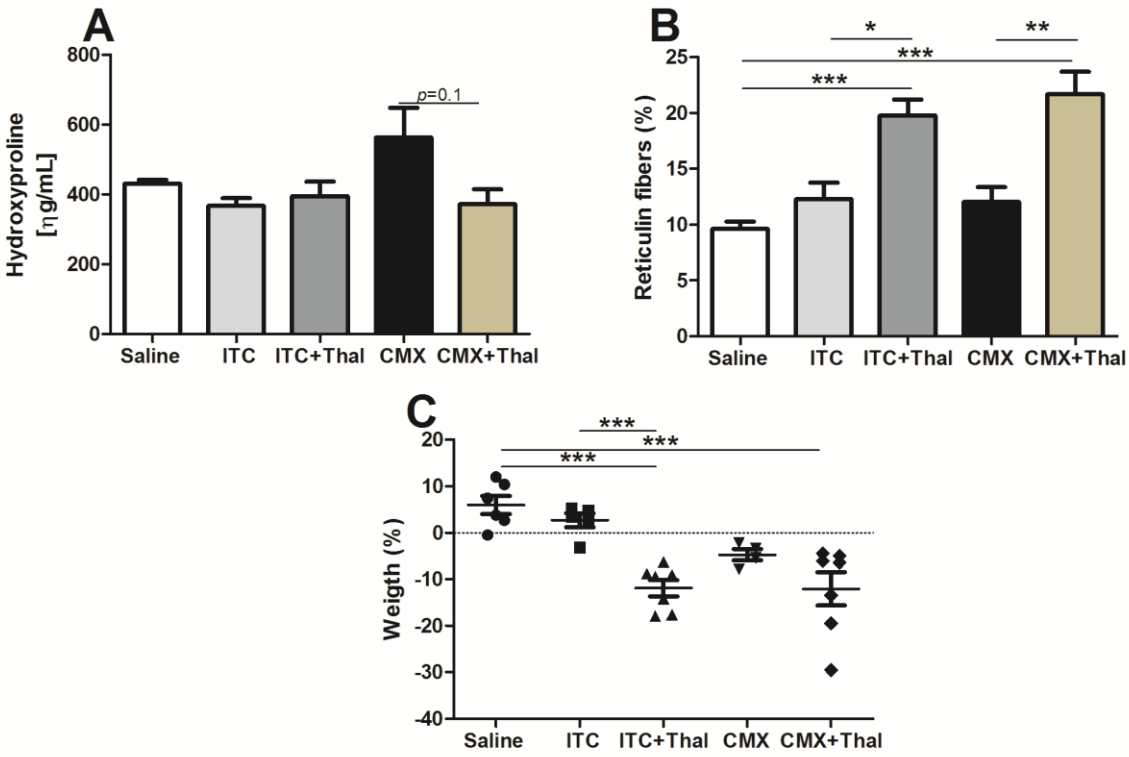


Figure 6. Evaluation of thalidomide (Thal) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) on clinical, histopathological and pulmonary fibrosis parameters. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the following therapeutic regimens were started: Thal/ITC, Thal/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) Quantification of hydroxyproline in the lungs. Values expressed in ηg/mL. B) Determination of the percentage of reticulin fibers in the lungs. C) Weight variation between the beginning and the end of the treatment. Data expressed as a percentage (%). ANOVA test with Tukey post-test; **p* <0.05; ***p* <0.01; ****p* <0.001, n=5-7.

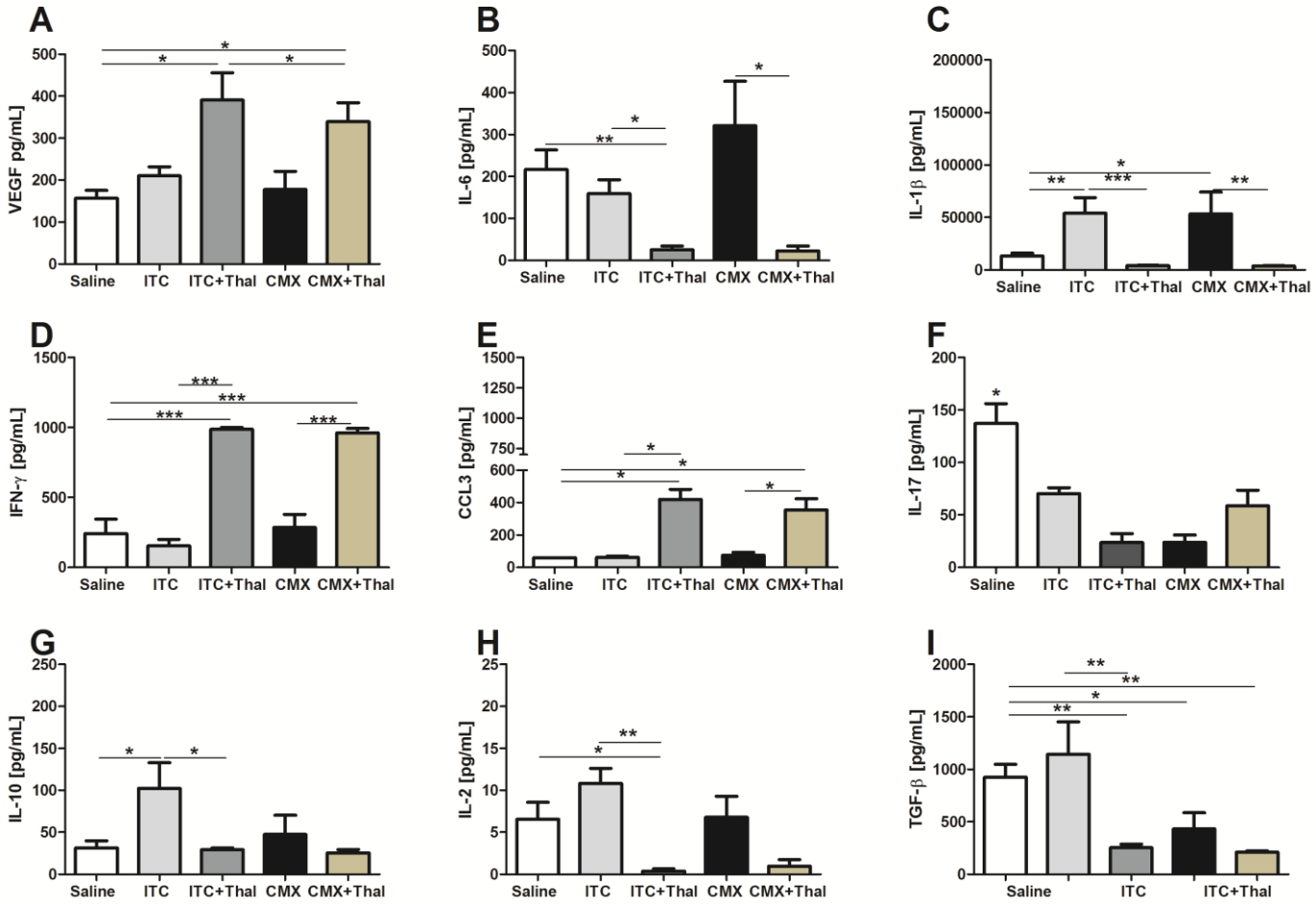
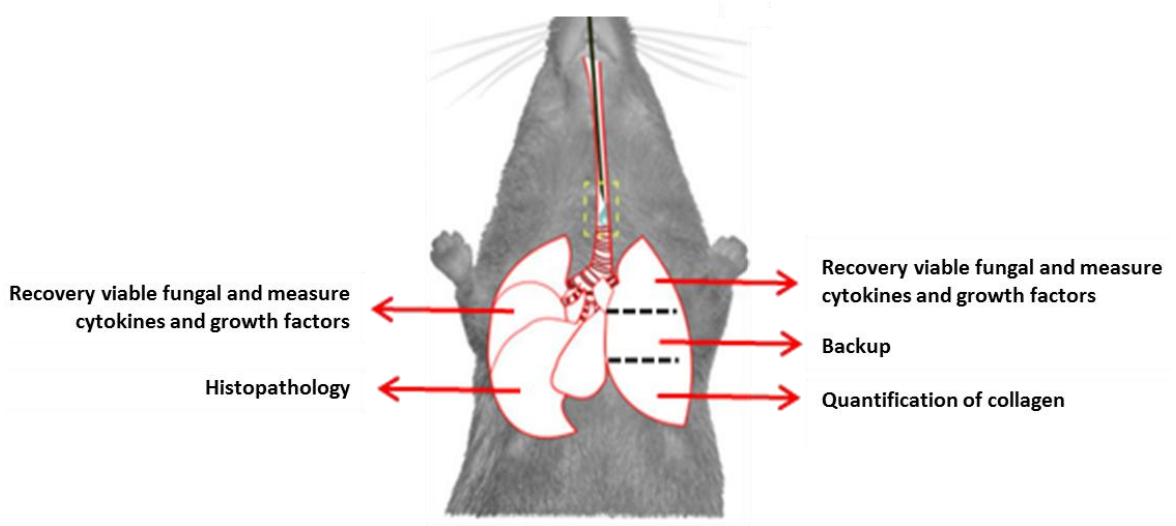
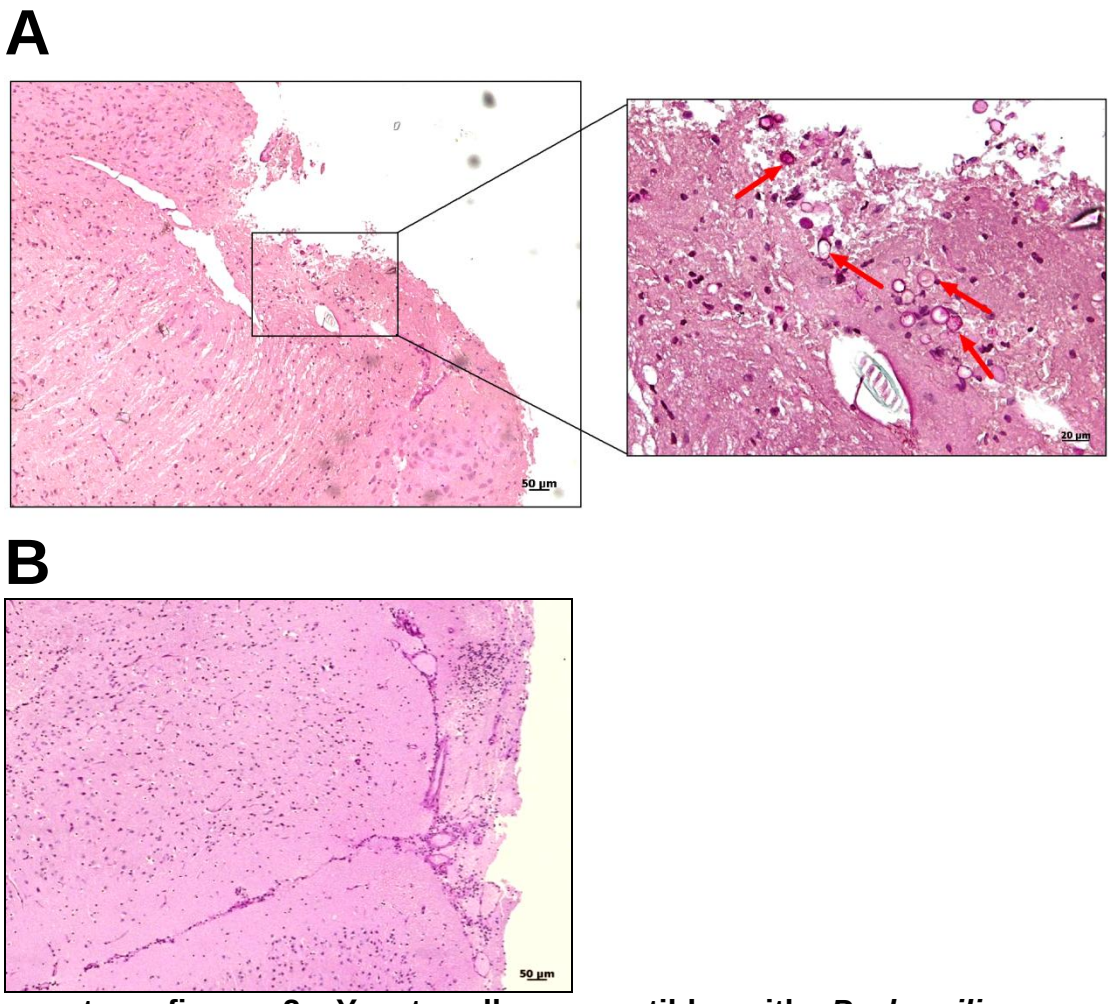


Figure 7. Influence of thalidomide (Thal) in combination with the antifungals itraconazole (ITC) and cotrimoxazole (CMX) in the quantification of inflammatory mediators and growth factors in the lungs. BALB/c mice were infected with P. brasiliensis and at the 8th week of infection the following therapeutic regimens were started: Thal/ITC, Thal/CMX and placebo. The mice were evaluated after 8 weeks of treatment. A) VEGF, B) IL-6, C) IL-1β, D) IFN-γ, E) CCL3, F) IL-17, G) IL-10, H) IL-2 and I) TGF-β. ANOVA test with Tukey post-test; *p <0.05; **p <0.01; ***p <0.001, n=5-7.



Supplementary Figure 1. **Collection of biological material: fragmentation of lungs** (Adapted Munder Antje, Wölbeling Florian, Kerber-Momot Tanja, Wedekind Dirk, Baumann Ulrich, Gulbins Erich, et al. Acute intratracheal Pseudomonas aeruginosa infection in cystic fibrosis mice is age-independent. *Respir Res* 2011;**12**:148. Doi: 10.1186/1465-9921-12-148.)



Supplementary figure 2. **Yeast cells compatible with *P. brasiliens* and inflammatory inffiltrate in brain**. BALB/c mice were infected with *P. brasiliensis* and at the 8th week of infection the Thal/CMX therapeutic regimen were initiated. Two mice were euthanized at 6th week of treatment, exhibiting neurological alterations. A) Yeast cells compatible with *P. brasiliens* (red arrows) in the brain. B) Inflammatory infiltrate in the brain. PAS staining.

REFERENCES

1 Teixeira Marcus M, Theodoro Raquel C, de Carvalho Maria JA, Fernandes Larissa, Paes Hugo C, Hahn Rosane C, et al. Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. *Mol Phylogenet Evol* 2009;**52**(2):273–83. Doi: 10.1016/j.ympev.2009.04.005.

2 Bocca Anamelia Lorenzetti, Amaral André Corrêa, Teixeira Marcus Melo, Sato Paula Keiko, Sato Paula, Shikanai-Yasuda Maria Aparecida, et al. Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues. *Future Microbiol* 2013;**8**(9):1177–91. Doi: 10.2217/fmb.13.68.

3 Mendes RP. The gamut of clinical manifestations. In: Franco M, Lacaz CS, Restrepo-Moreno A, Del Negro G, editors. Paracoccidioidomycosis. Boca Raton: CRC Press; 1994. p. 233-58

4 Tobón AM, Agudelo CA, Osorio ML, Alvarez DL, Arango M, Cano LE, et al. Residual pulmonary abnormalities in adult patients with chronic paracoccidioidomycosis: prolonged follow-up after itraconazole therapy. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2003;**37**(7):898–904. Doi: 10.1086/377538.

5 Campos EP, Cataneo AJ. [Pulmonary function in 35 patients with paracoccidioidomycosis]. *Rev Inst Med Trop Sao Paulo* 1986;**28**(5):330–6.

6 Campos EP, Padovani CR, Cataneo AM. [Paracoccidioidomycosis: radiologic and pulmonary study in 58 cases]. *Rev Inst Med Trop Sao Paulo* 1991;**33**(4):267–76.

7 Venturini James, Cavalcante Ricardo Souza, Golim Márjorie de Assis, Marchetti Camila Martins, Azevedo Priscila Zacarias de, Amorim Bárbara Casella, et al. Phenotypic and functional evaluations of peripheral blood monocytes from chronic-form paracoccidioidomycosis patients before and after treatment. *BMC Infect Dis* 2014;**14**:552. Doi: 10.1186/s12879-014-0552-x.

8 Cock AM, Cano LE, Vélez D, Aristizábal BH, Trujillo J, Restrepo A. Fibrotic sequelae in pulmonary paracoccidioidomycosis: histopathological aspects in BALB/c mice infected with viable and non-viable paracoccidioides brasiliensis propagules. *Rev Inst Med Trop Sao Paulo* 2000;**42**(2):59–66.

9 Araujo Stanley de Almeida. Contribuição ao estudo anátomo-clínico da Paracoccidioiodomicose em Minas Gerais. meio século de experiência - avaliação das necrópsias realizadas no período compreendido entre 1944 até 1999, no departamento de anatomia patológica e medicina legal, da Faculdade de Medicina da Universidade Federal de Minas Gerais. Available at http://www.bibliotecadigital.ufmg.br/dspace/handle/1843/BUOS-8KVLVK. Accessed June 21, 2017, 2011.

10 Naranjo Tonny W, Lopera Damaris E, Diaz-Granados Lucy R, Duque Jhon J, Restrepo Angela M, Cano Luz E. Combined itraconazole-pentoxifylline treatment promptly reduces lung fibrosis induced by chronic pulmonary paracoccidioidomycosis in mice. *Pulm Pharmacol Ther* 2011;**24**(1):81–91. Doi: 10.1016/j.pupt.2010.09.005.

11 Park MK, Fontana JR, Babaali H, Gilbert-McClain LI, Stylianou M, Joo J, et al. Steroid-Sparing effects of pentoxifylline in pulmonary sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis Off J WASOG World Assoc Sarcoidosis Granulomatous Disord* 2009;**26**(2):121–31.

12 Zabel P, Entzian P, Dalhoff K, Schlaak M. Pentoxifylline in treatment of sarcoidosis. *Am J Respir Crit Care Med* 1997;**155**(5):1665–9. Doi: 10.1164/ajrccm.155.5.9154873.

13 Wen Wei Xiong, Lee Siang Yin, Siang Rafaella, Koh Rhun Yian. Repurposing Pentoxifylline for the Treatment of Fibrosis: An Overview. *Adv Ther* 2017;**34**(6):1245–69. Doi: 10.1007/s12325-017-0547-2.

14 Neuner P, Klosner G, Schauer E, Pourmojib M, Macheiner W, Grünwald C, et al. Pentoxifylline in vivo down-regulates the release of IL-1 beta, IL-6, IL-8 and tumour necrosis factor-alpha by human peripheral blood mononuclear cells. *Immunology* 1994;**83**(2):262–7.

15 Mandell GL. Cytokines, phagocytes, and pentoxifylline. *J Cardiovasc Pharmacol* 1995;**25 Suppl 2**:S20-22.

16 Berman B, Duncan MR. Pentoxifylline inhibits normal human dermal fibroblast in vitro

proliferation, collagen, glycosaminoglycan, and fibronectin production, and increases collagenase activity. *J Invest Dermatol* 1989;**92**(4):605–10.
17 Gilhar A, Grossman N, Kahanovicz S, Reuveni H, Cohen S, Eitan A. Antiproliferative effect of pentoxifylline on psoriatic and normal epidermis. In vitro and in vivo studies. *Acta Derm Venereol* 1996;**76**(6):437–41.
18 Kawamura Kodai, Ichikado Kazuya, Yasuda Yuko, Anan Keisuke, Suga Moritaka. Azithromycin for idiopathic acute exacerbation of idiopathic pulmonary fibrosis: a retrospective single-center study. *BMC Pulm Med* 2017;**17**. Doi: 10.1186/s12890-017-0437-z.
19 Southern Kevin W, Barker Pierre M, Solis-Moya Arturo, Patel Latifa. Macrolide antibiotics for cystic fibrosis. *Cochrane Database Syst Rev* 2012;**11**:CD002203. Doi: 10.1002/14651858.CD002203.pub4.
20 Vanaudenaerde BM, Wuyts WA, Geudens N, Dupont LJ, Schoofs K, Smeets S, et al. Macrolides Inhibit IL17-induced IL8 and 8-isoprostane Release from Human Airway Smooth Muscle Cells. *Am J Transplant* 2007;**7**(1):76–82. Doi: 10.1111/j.1600-6143.2006.01586.x.
21 Wuyts WA, Willems S, Vos R, Vanaudenaerde BM, De Vleeschauwer SI, Rinaldi M, et al. Azithromycin reduces pulmonary fibrosis in a bleomycin mouse model. *Exp Lung Res* 2010;**36**(10):602–14. Doi: 10.3109/01902148.2010.492895.
22 Khan AA, Slifer TR, Araujo FG, Remington JS. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. *Int J Antimicrob Agents* 1999;**11**(2):121–32.
23 Zhou Shuang, Wang Fengfei, Hsieh Tze-Chen, Wu Joseph M, Wu Erxi. Thalidomide-a notorious sedative to a wonder anticancer drug. *Curr Med Chem* 2013;**20**(33):4102–8.
24 Choe Jung-Yoon, Jung Hyun-Joo, Park Ki-Yeun, Kum Yoon-Seup, Song Gwan Gyu, Hyun Dae-Sung, et al. Anti-fibrotic effect of thalidomide through inhibiting TGF-beta-induced ERK1/2 pathways in bleomycin-induced lung fibrosis in mice. *Inflamm Res Off J Eur Histamine Res Soc Al* 2010;**59**(3):177–88. Doi: 10.1007/s00011-009-0084-9.
25 Tabata Chiharu, Tabata Rie, Kadokawa Yoshio, Hisamori Shigeo, Takahashi Meiko, Mishima Michiaki, et al. Thalidomide prevents bleomycin-induced pulmonary fibrosis in mice. *J Immunol Baltim Md 1950* 2007;**179**(1):708–14.
26 Horton Maureen R, Santopietro Victoria, Mathew Leena, Horton Karen M, Polito Albert J, Liu Mark C, et al. Thalidomide for the treatment of cough in idiopathic pulmonary fibrosis: a randomized trial. *Ann Intern Med* 2012;**157**(6):398–406. Doi: 10.7326/0003-4819-157-6-201209180-00003.
27 Scavone Renata, Burger Eva. Paracoccidioidomycosis: reduction in fungal load and abrogation of delayed-type hypersensitivity anergy in susceptible inbred mice submitted to therapy with trimethoprim-sulfamethoxazole. *Med Microbiol Immunol (Berl)* 2004;**193**(1):53–9. Doi: 10.1007/s00430-003-0185-y.
28 Shikanai-Yasuda Maria Aparecida, Telles Filho Flávio de Queiroz, Mendes Rinaldo Pôncio, Colombo Arnaldo Lopes, Moretti Maria Luiza. [Guidelines in paracoccidioidomycosis]. *Rev Soc Bras Med Trop* 2006;**39**(3):297–310.
29 LIU XUEJUN, QIAN LI, NAN HAOYU, CUI MIAO, HAO XIAOYAN, DU YUFENG. Function of the transforming growth factor-β1/c-Jun N-terminal kinase signaling pathway in the action of thalidomide on a rat model of pulmonary fibrosis. *Exp Ther Med* 2014;**7**(3):669–74. Doi: 10.3892/etm.2013.1457.
30 Karlmark Karlin Raja, Weiskirchen Ralf, Zimmermann Henning W, Gassler Nikolaus, Ginhoux Florent, Weber Christian, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009;**50**(1):261–74. Doi: 10.1002/hep.22950.
31 Lopera Damaris, Naranjo Tonny W, Cruz Oswaldo G, Restrepo Angela, Cano Luz Elena, Lenzi Henrique Leonel. Structural and topographic dynamics of pulmonary histopathology and local cytokine profiles in Paracoccidioides brasiliensis conidia-infected mice. *PLoS Negl Trop Dis* 2011;**5**(7):e1232. Doi: 10.1371/journal.pntd.0001232.
32 Tuder RM, el Ibrahim R, Godoy CE, De Brito T. Pathology of the human pulmonary paracoccidioidomycosis. *Mycopathologia* 1985;**92**(3):179–88.
33 de Castro Lívia Furquim, Ferreira Maria Carolina, da Silva Rosiane Maria, Blotta Maria Heloisa de

Souza Lima, Longhi Larissa Nara Alegrini, Mamoni Ronei Luciano. Characterization of the immune response in human paracoccidioidomycosis. *J Infect* 2013;**67**(5):470–85. Doi: 10.1016/j.jinf.2013.07.019.

34 Benard G, Romano CC, Cacere CR, Juvenale M, Mendes-Giannini MJ, Duarte AJ. Imbalance of IL-2, IFN-gamma and IL-10 secretion in the immunosuppression associated with human paracoccidioidomycosis. *Cytokine* 2001;**13**(4):248–52. Doi: 10.1006/cyto.2000.0824.

35 Mamoni Ronei Luciano, Blotta Maria Heloisa Souza Lima. Flow-cytometric analysis of cytokine production in human paracoccidioidomycosis. *Cytokine* 2006;**35**(3):207–16. Doi: 10.1016/j.cyto.2006.08.005.

36 Mamoni RL, Nouér SA, Oliveira SJ, Musatti CC, Rossi CL, Camargo ZP, et al. Enhanced production of specific IgG4, IgE, IgA and TGF-β in sera from patients with the juvenile form of paracoccidioidomycosis. *Med Mycol* 2002;**40**(2):153–9. Doi: 10.1080/mmy.40.2.153.159.

37 Oliveira Sara J, Mamoni Ronei L, Musatti Chloé C, Papaiordanou Priscilla MO, Blotta Maria Heloisa SL. Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls. *Microbes Infect* 2002;**4**(2):139–44. Doi: 10.1016/S1286-4579(01)01521-0.

38 Cavalcante Ricardo de Souza, Sylvestre Tatiane Fernanda, Levorato Adriele Dandara, de Carvalho Lídia Rachel, Mendes Rinaldo Poncio. Comparison between itraconazole and cotrimoxazole in the treatment of paracoccidiodomycosis. *PLoS Negl Trop Dis* 2014;**8**(4):e2793. Doi: 10.1371/journal.pntd.0002793.

39 Queiroz-Telles Flavio, Goldani Luciano Z, Schlamm Haran T, Goodrich James M, Espinel-Ingroff Ana, Shikanai-Yasuda Maria A. An open-label comparative pilot study of oral voriconazole and itraconazole for long-term treatment of paracoccidioidomycosis. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2007;**45**(11):1462–9. Doi: 10.1086/522973.

40 Emiralioğlu Nagehan, Öztürk Zeynelabidin, Yalçın Ebru, Doğru Deniz, Özçelik Uğur, Kiper Nural. Long term azithromycin therapy in patients with cystic fibrosis. *Turk J Pediatr* 2016;**58**(1):34–40.

41 Arai Hideyuki, Furusu Akira, Nishino Tomoya, Obata Yoko, Nakazawa Yuka, Nakazawa Masayuki, et al. Thalidomide prevents the progression of peritoneal fibrosis in mice. *Acta Histochem Cytochem* 2011;**44**(2):51–60. Doi: 10.1267/ahc.10030.

42 Banach Marta, Juranek Judyta K, Zygulska Aneta L. Chemotherapy-induced neuropathies-a growing problem for patients and health care providers. *Brain Behav* 2017;**7**(1):e00558. Doi: 10.1002/brb3.558.

43 Cavaletti G, Beronio A, Reni L, Ghiglione E, Schenone A, Briani C, et al. Thalidomide sensory neurotoxicity: a clinical and neurophysiologic study. *Neurology* 2004;**62**(12):2291–3.

44 Plasmati R, Pastorelli F, Cavo M, Petracci E, Zamagni E, Tosi P, et al. Neuropathy in multiple myeloma treated with thalidomide: a prospective study. *Neurology* 2007;**69**(6):573–81. Doi: 10.1212/01.wnl.0000267271.18475.fe.

45 Pedroso Vinicius Sousa Pietra, Vilela Márcia Carvalho, Santos Patrícia Campi, Cisalpino Patrícia Silva, Rachid Milene Alvarenga, Teixeira Antônio Lúcio. Traffic of leukocytes and cytokine up-regulation in the central nervous system in a murine model of neuroparacoccidioidomycosis. *Mycopathologia* 2013;**176**(3–4):191–9. Doi: 10.1007/s11046-013-9679-3.

46 Heinrichs Daniel, Berres Marie-Luise, Nellen Andreas, Fischer Petra, Scholten David, Trautwein Christian, et al. The chemokine CCL3 promotes experimental liver fibrosis in mice. *PloS One* 2013;**8**(6):e66106. Doi: 10.1371/journal.pone.0066106.

47 Hamada Naoki, Kuwano Kazuyoshi, Yamada Mizuho, Hagimoto Naoki, Hiasa Kenichi, Egashira Kensuke, et al. Anti-vascular endothelial growth factor gene therapy attenuates lung injury and fibrosis in mice. *J Immunol Baltim Md 1950* 2005;**175**(2):1224–31.

48 Chaudhary NI, Roth GJ, Hilberg F, Müller-Quernheim J, Prasse A, Zissel G, et al. Inhibition of PDGF, VEGF and FGF signalling attenuates fibrosis. *Eur Respir J* 2007;**29**(5):976–85. Doi: 10.1183/09031936.00152106.

Conclusões

5. Conclusões

A pentoxifilina quando associada ao itraconazol apresentou efeito antifibrótico, entretanto quando utilizada com o cotrimoxazol não se mostrou eficiente. A azitromicina mostrou-se mais eficaz na combinação com o cotrimoxazol. Os mecanismos envolvidos estavam relacionados a diminuição da produção de TGF-β e aumento de IL-10. O tratamento com talidomida não foi satisfatório nas associações com itraconazol e cotrimoxazol, pois não apresentou efeito antifibrótico, além de ter agravado a doença nos animais tratados com cotrimoxazol.

A intensificação dos quadros de fibrose em indivíduos tratados com os diferentes esquemas terapêuticos não eficazes, estiveram associados a níveis elevados de CCL3 e VEGF nos pulmões desses animais.

Assim, os resultados obtidos abrem novas perspectivas para aplicação terapêutica das drogas avaliadas na paracoccidioidomicose pulmonar.