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Original Article

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Efficacy of a poly-aggregated formulation of amphotericin B in treating systemic sporotrichosis caused by *Sporothrix brasiliensis*

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Abstract

In severe cases of sporotrichosis, it is recommended to use amphotericin B deoxycholate (D-AMB) or its lipid formulations and/or in association with itraconazole (ITC). Our aim was to evaluate the antifungal efficacy of a poly-aggregated amphotericin B (P-AMB), a nonlipid formulation, compared with D-AMB on systemic sporotrichosis caused by *Sporothrix brasiliensis*. *In vitro* assays showed that *Sporothrix schenckii sensu stricto* and *S. brasiliensis* yeast clinical isolates were susceptible to low concentrations of P-AMB and D-AMB. Although P-AMB presented a higher minimal inhibitory concentration (MIC) compared to D-AMB, its cytotoxic effect on renal cells and erythrocytes was lower. For the *in vivo* assays, male BALB/c mice were intravenously infected with *S. brasiliensis* yeasts, and P-AMB or D-AMB was administered 3 days post-infection. The efficacy of five therapeutic regimens was tested: intravenous monotherapy with P-AMB or D-AMB, intravenous pulsed-therapy with P-AMB or D-AMB, and intravenous therapy with P-AMB, followed by oral ITC. These treatments increased murine survival and controlled the fungal burden in the liver, spleen, lungs, and kidneys. However, only D-AMB monotherapy or the pulsed-therapies with D-AMB or P-AMB led to 100% survival of the mice 45

days post-infection; only pulsed administration of D-AMB was able to control the fungal load in all organs 45 days post-infection. Accordingly, the histopathological findings showed reductions in the fungal burden and inflammatory reactions in these treatment regimens. Together, our results suggest that the P-AMB formulation could be considered as an alternative drug to D-AMB for treating disseminated sporotrichosis.

Key words: sporotrichosis, *Sporothrix brasiliensis*, poly-aggregated AMB, amphotericin B, sporotrichosis mouse model, antifungal drugs.

Introduction

Sporotrichosis is a chronic subcutaneous mycosis caused by the pathogenic species of the *Sporothrix schenckii* complex.[1] This mycosis has several clinical manifestations, but its severe forms are generally associated with an immunocompromised host or observed in susceptible animals.[2] Epidemiologically, in Brazil, sporotrichosis is frequently reported in humans and domestic cats, and interestingly, the zoonotic transmission is related to *Sporothrix brasiliensis*.[2,3] This new emerging species is also associated with severe cases of the disease.[4–6] *In vivo* studies have revealed that *S. brasiliensis* isolates are more virulent than *Sporothrix schenckii sensu stricto* and that they are able to disseminate from the primary cutaneous lesion.[7,8] In addition, *S. brasiliensis* clinical isolates showed lower antifungal susceptibility *in vitro* in relation to S. *schenckii s. str.*[9,10]

The current protocol for sporotrichosis treatment follows the guidelines of the *American Society of Infectious Diseases* recommending the use of itraconazole (ITC) as the gold standard drug for lymphocutaneous/cutaneous manifestation; in contrast, amphotericin B deoxycholate (D-AMB) or its lipid formulations in combination or not with ITC are alternatives for severe forms of this disease.[11] Nevertheless, improvements in the therapeutic protocols should consider the features of the new cryptic species of *S. schenckii* complex, their antifungal susceptibility pattern, and the host immune status.[12–15] Furthermore, our group has recently reported that ITC demonstrated low efficiency in treating *S. brasiliensis* disseminated infection in a murine model; in contrast D-AMB followed with ITC showed efficiency to control the infection in the murine model.[9]

Among antifungals with activity against *Sporothrix* spp., D-AMB remains as the most effective treatment for severe cases of sporotrichosis,[9,11] despite its acute and chronic side effects, mainly the nephrotoxicity.[16] The lipid formulations of AMB (Ambisome®, Abelcet®, and Amphotec®) demonstrate better tolerance and good efficacy for several fungal infections,[5,17–21] but the higher treatment cost for these formulations prevents their widespread use, primarily in some developing countries. For this reason, new delivery systems of AMB, including lipid-free formulations, have been developed in the last years.[22–26] Among various formulations, the poly-aggregated form of AMB (P-AMB) has been tested in murine models of candidiasis and leishmaniasis, demonstrating good therapeutic efficacy and lower toxicity compared with D-AMB.[24–26]

Based on these background data, the aim of this work was to evaluate the *in vitro* susceptibility of *S. brasiliensis* strains to P-AMB, cytotoxicity, and its efficacy in the murine model of disseminated sporotrichosis caused by *S. brasiliensis* comparing with D-AMB.

Methods

Fungal strains

In this study, three *Sporothrix schenckii sensu stricto* strains (ATCC MYA-4821, ATCC MYA-4820, and SB02) and three *Sporothrix brasiliensis* strains (ATCC MYA-4823, ATCC MYA-4824, and FMR8337) were used for the *in vitro* antifungal susceptibility assay. *Sporothrix brasiliensis* ATCC MYA-4823 strain, which was obtained from feline sporotrichosis, was considered to be the highly virulent isolate by our group[8] and was chosen for the *in vivo* assays.

The mycelial form of *Sporothrix* spp. was obtained and maintained on Sabouraud dextrose agar (SDA, Becton, Dickinson & Company, Sparks, USA) at 28 °C. For conversion to its yeast form, the mycelial form was subcultured twice on brain–heart infusion broth (BHI, Becton, Dickinson and Company, USA) at 36 °C under orbital agitation for 7 days. Then, the total yeast conversion was confirmed by observing the yeasts with light microscopy.

Antifungal drugs

The poly-aggregated form of amphotericin B (P-AMB) was obtained, as previously described by Sánchez-Brunete et al.[22] For the *in vitro* assays, itraconazole (ITC, Sigma Chemical Co., Missouri, USA), amphotericin B deoxycholate (D-AMB, Sigma Chemical Co., Missouri, USA) and poly-aggregated form of AMB (P-AMB) were diluted in dimethyl sulfoxide (DMSO) to obtain concentrations at 1,600 μg/ml. For the *in vivo* assays, D-AMB (Cristalia, Brazil) and P-AMB were diluted in 5% glucose sterile solution; and ITC (Sporanox®, from Janssen-Cilag Pharmaceuticals Inc.,

Australia) was diluted in sterile phosphate-buffered saline (PBS).

In vitro antifungal susceptibility testing

The broth microdilution assays were performed using methodologies adapted from protocols published by the Clinical Laboratory Standard Institute for the mycelial form[27] and for yeast[28], as described by Borba-Santos et al.[10] Serial twofold dilutions of the antifungal drugs (P-AMB, D-AMB, or ITC) were performed in RPMI 1640 medium buffered with 3-(N-morpholino)propanesulfonic acid 0.16 M, pH 7.0 (both from Sigma Chemical Co., Missouri, USA) and supplemented (yeast) or not (mycelial form) with 2% glucose, into 96-well microtiter trays to obtain final concentrations of 0.03–16 μg/ml for all antifungals. Then, yeast or conidia cells were added to each well to obtain a final concentration of 0.5–2.5 $\times$ 10^3 cfu/ml and 0.4–5 $\times$ 10^4 cfu/ml, respectively. The microtiter trays were incubated at 35°C for 5 days in a dark and humid chamber with 5% CO_2. Then, the minimal inhibitory concentration (MIC) of antifungal was determined by visual inspection in an inverted optical microscope, and it was defined as the lowest concentration that inhibits 90–100% of fungal growth in relation to the untreated cells. The quality control strain used in the assays was *Candida parapsilosis* ATCC 22019 as described in the CLSI documents.[27,28] After MIC determination, an aliquot of 10 μl of fungal cells treated with inhibitory concentrations were subcultured onto free-drug SDA surface at 35°C for 7 days. The minimal fungicidal concentration (MFC) value was defined as the lowest concentration that showed no fungal growth.[29]

P-AMB cytotoxicity and antifungal selective index (SI) toward *Sporothrix* spp.

To evaluate the selectivity of P-AMB towards *Sporothrix* spp., the cytotoxicity assay was performed on mammalian cells (monkey renal cells [LLC-MK2] and human red blood cells [RBC]) using 1–100 μg/ml, as previously described, and compared with D-AMB.[9] CC_{50} (a drug concentration yielding 50% cytotoxicity to LLC-MK2 cells) and HA_{50} (a drug concentration yielding 50% of hemolysis of RBC) values were determined, and the antifungal drugs selectivity indexes (SIs) were calculated according to the following formula: SI = HA_{50} or CC_{50}/yeast MIC.[9]

Murine model of disseminated sporotrichosis

In sum, 8 to 10-week old BALB/c male mice weighing ~25 g were maintained in accordance with the National Institutes of Health Animal Care Guidelines.[30] The Ethics Committee of the Instituto de Biologia Roberto Alcantara Gomes (State University of Rio de Janeiro, Brazil) previously approved all study procedures (process number CEA/027/2010).

The murine model of disseminated sporotrichosis described by Ishida et al.[9] was used in this study. Briefly, the mice were intravenously inoculated through the lateral tail vein with 10^5 yeast cells of the *S. brasiliensis* ATCC MYA-4823 strain suspended in 0.1 ml of sterile PBS. Uninfected groups received the same vehicle volume. The inoculum viability was verified by plating a volume of the yeast suspensions on SDA medium immediately after inoculation, and the number of cfu was determined after 7 days of incubation at 36 °C. The yeast form of the *S. brasiliensis* was used to simulate the zoonotic transmission of sporotrichosis.

In vivo antifungal treatments and efficacy analysis

The antifungal treatments started after 3 days of the yeast infection, and five therapeutic regimens were assayed: i) monotherapy with D-AMB 1 mg/kg/day (protocol 1) or P-AMB 3 mg/kg/day (protocol 2) administered intravenously for 7 consecutive days (D-AMB and P-AMB groups, respectively); ii) pulsed-therapy with D-AMB 1 mg/kg/day (protocol 3) or P-AMB 3 mg/kg/day (protocol 4) administered intravenously for 7 consecutive days, followed by a 7-day interval, and after drug administration for 7 consecutive days, using the same dose (D-AMB pulse and P-AMB pulse groups, respectively); and iii) P-AMB 3 mg/kg/day administered intravenously for 7 consecutive days followed by ITC 37.5 mg/kg (protocol 5) administered orally twice a day for 20 days (P-AMB/ITC group). The antifungal treatments were compared with a group of untreated animals (NT group).

Survival curve

Treated and untreated mice (9 animals in each group) were monitored daily for at least 45 days post-infection (p.i.) to determine the survival curve and other parameters (e.g., animal weight, physical condition and behavior) were also monitored.

Fungal load estimation

Organs (i.e., kidneys, liver, lungs, and spleen) from treated and untreated animals were removed aseptically and weighed after 12, 30, or 45 days p.i. (n = 6 mice in each group/time). All organs were macerated in sterile PBS, and 100 μl of homogenate was plated onto SDA plates with 50 μg/ml of chloramphenicol (Sigma Chemical Co., USA)

Table 1. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values of poly-aggregated amphotericin B (P-AMB), amphotericin B deoxycholate (D-AMB), and itraconazole (ITC) toward mycelia and yeast forms of *S. schenckii s. str.* and *S. brasiliensis* strains.

	Mycelial phase						Yeast phase					
	P-AMB		D-AMB		ITC		P-AMB*		D-AMB*		ITC	
Strains	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
S. brasiliensis MYA-4824	8	>16	4	8	4	>16	2	4	0.25	0.5	0.5	8
S. brasiliensis MYA-4823	2	>16	0.5	2	4	>16	1	16	0.5	0.5	0.5	16
S. brasiliensis FMR 8337	1	2	0.5	0.5	2	>16	1	1	0.25	0.5	1	>16
S. schenckii MYA-4821	1	>16	0.5	16	1	>16	4	16	0.5	0.5	1	4
S. schenckii MYA-4820	16	>16	1	1	>16	>16	4	4	0.5	8	1	2
S. schenckii SB02	2	16	0.5	16	0.5	>16	2	2	0.5	0.5	0.5	0.5

The MIC and MFC values are provided in μg/ml.
*$p = 0.006$.

and incubated at 28°C for 7 days to count the cfu to determine the cfu number per gram of tissue (cfu/g).

Histopathological analysis

Mice from all treatment groups were euthanized after 12 or 45 days p.i. ($n = 6$ mice in each group/time) to analyze the histological effects on the organs after the antifungal treatment. The kidneys and liver were removed aseptically, fixed in 10% formaldehyde in PBS, and prepared for histopathological examination. Assessment of inflammatory cell infiltration was performed in hematoxylin and eosin (HE)-stained slides using an arbitrary semi-quantitative scale that ranged from 0 to 3 for neutrophils (0 = none, 1 = some without abscesses, 2 = small abscesses, and 3 = large abscesses) and from 0 to 4 for mononuclear phagocyte cells (0 = none, 1 = without granulomas, 2 = poorly formed granulomas, 3 = well-formed simple granulomas, and 4 = well-formed complex granulomas) (also see Supplementary Fig. S1). The fungal burden was analyzed in Gomori-Grocott's methenamine-silver (GMS) and periodic acid-Schiff (PAS)-stained slides using an arbitrary semi-quantitative scale ranging from 0 to 4 (0, none; 1, limited; 2, average; 3, high; 4, very high).[9]

Statistical analysis

GraphPad Prism software (version 6.0, La Jolla, California, USA) was used for the statistical analysis. The comparison of the antifungal MIC values was performed using the Mann–Whitney *U* test. The survival curve of the animals was analyzed using the Wilcoxon rank test, and the differences between fungal loads in the organs from mice submitted by antifungal treatments were analyzed with one-way ANOVA. A *P* value less than .05 was considered to be significant.

Results

P-AMB is active against *S. schenckii s. str.* and *S. brasiliensis* and less cytotoxic than D-AMB.

The *in vitro* antifungal activity of P-AMB was assessed according to the MIC and MFC values determined for three isolates of *S. schenckii s. str.* and *S. brasiliensis* (Table 1) and then compared to the D-AMB and ITC. For 100% and ~70% of *Sporothrix* spp. strains in the yeast and mycelial forms, respectively, were inhibited by antifungals in low concentrations (MIC $\leq$ 4 μg/ml) (Table 1). No statistically significant difference was observed between the mycelial and yeast form MIC values for all antifungals ($P > .05$). P-AMB was active against *Sporothrix* spp. isolates in both fungal forms (MIC $\leq$ 4 μg/ml), except on *S. brasiliensis* ATCC MYA-4824 and *S. schenckii* ATCC MYA-4820 in the mycelia phase; in addition, it was more active against yeasts presenting fungicidal effect (Table 1). D-AMB presented fungicidal action against yeast and mycelia forms; in contrast ITC showed fungistatic effect for both fungal phases, except for yeast form of *S. schenckii s. str.* isolates (Table 1).

The yeast phase of the virulent clinical isolate of *S. brasiliensis* (ATCC MYA-4823), selected for the *in vivo* experiments, was considered to be susceptible to P-AMB (MIC = 1 μg/ml and MFC = 16 μg/ml) and to D-AMB (MIC = 0.5 mg/ml and MFC = 0.5 μg/ml) (Table 1).

Although P-AMB has been considered to be less active than D-AMB on yeast form of *Sporothrix* spp. isolates ($P = .006$), P-AMB was also less cytotoxic than D-AMB

Table 2. Cytotoxicity and selective indexes (SIs) of poly-aggregated amphotericin B (P-AMB) and amphotericin B deoxycholate (D-AMB).

Antifungal	MIC_y median (μg/ml)	LLC-MK_2 cells		Red blood cells	
		CC_{50} (μg/ml)	SI	HA_{50} (μg/ml)	SI
P-AMB	2	26.5	13.25	13.25	6.62
D-AMB	0.5	6.25[a]	12.5	6.25[a]	12.5

CC_{50}, drug concentration yielding 50% cytotoxicity in monkey renal cells (LLC-MK2); HA_{50}, drug concentration yielding 50% hemolytic activity in human red blood cells (RBC); MIC_y median, the median minimal inhibitory concentration values toward yeasts; SI_y, selectivity index for the yeasts; [a]Data obtained by Ishida et al. [9].

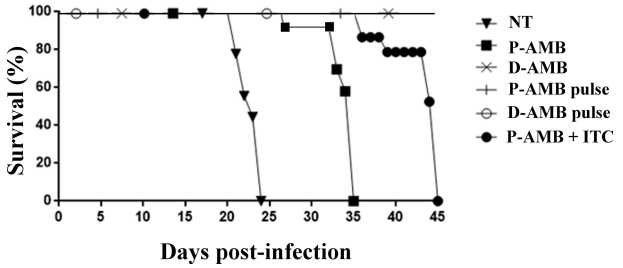

Figure 1. Survival rate of the BALB/c male mice infected with *Sporothrix brasiliensis* yeasts untreated (NT) and treated with the following antifungal therapies: (a) monotherapy with 1 mg/kg/day of amphotericin B deoxycholate (D-AMB) or 3 mg/kg/day of poly-aggregated amphotericin B (P-AMB), intravenously, for 7 days; (b) pulsed-therapy with D-AMB 1 mg/kg/day or P-AMB 3 mg/kg/day administered intravenously for 7 consecutive days, followed by a 7-day interval, and a new round of drug administration for 7 consecutive days using the same dose (D-AMB pulse and P-AMB pulse); (c) 3 mg/kg/day of P-AMB for 7 days followed by 75 mg/kg/day of itraconazole (37.5 mg/kg b.i.d. orally) up to 30 days post-infection (P-AMB/ITC). $n = 9$ mice in each untreated and treated group. All antifungal therapies were statistically different of untreated group ($P < .01$).

(Table 2). P-AMB cytotoxic effects of 50% on LLC-MK_2 cells and on RBC were observed in concentrations of 26.5 μg/ml and 13.25 μg/ml, respectively (Table 2), 2–4 times less than the D-AMB cytotoxic effect; and P-AMB showed selectivity to yeast cells similar to the D-AMB (Table 2).

Efficacy of P-AMB and D-AMB in treating a murine disseminated infection caused by *S. brasiliensis*

All untreated animals succumbed to *S. brasiliensis* infection 24 days p.i. (Fig. 1). The P-AMB monotherapy and the combination of P-AMB and ITC significantly prolonged the survival rate of the mice ($P < .01$), but the animals succumbed at 35 and 44 days p.i., respectively. Interestingly, the monotherapy with D-AMB and the pulsed-therapy with D-AMB or P-AMB showed the highest efficacy, leading to 100% mice survival up to 45 days p.i. ($P < .001$) (Fig. 1). All infected animals suffered from weight loss and were listless; they presented with shallow breathing and reduced food and water intake; however, the untreated group showed prominent effects as early as 12 days p.i. Additionally, about 10% of animals treated with D-AMB died by convulsion immediately after the intravenous administration; in contrast, this behavior was not observed when the mice were administered with intravenous P-AMB.

The fungal burden in selected organs of mice infected with *S. brasiliensis* yeasts was estimated as the number of fungal colonies per gram of tissue (cfu/g) (Fig. 2). On the 12th day p.i., the monotherapy with D-AMB or pulsed D-AMB controlled the fungal load in all organs tested (liver, spleen, kidneys, and lungs) compared to the NT group ($P < .05$) (Fig. 2). Surprisingly, the monotherapy with P-AMB or P-AMB pulse therapy significantly inhibited the fungal load only in the liver compared to the NT group ($P < .05$). The therapeutic regimen with P-AMB followed by ITC (P-AMB/ITC) was also efficient at significantly controlling the fungal load in the liver and lungs on the 12th day p.i. ($P < .05$) (Fig. 2). Among the antifungal treatments, P-AMB monotherapy and P-AMB/ITC could not control or reduce the fungal load in all organs, and all animals succumbed before 45 days p.i.; however, the D-AMB monotherapy and D-AMB or P-AMB pulse therapy showed the most effective therapeutic scheme up to 45 days p.i.; these therapies were able to control the fungal load when compared with previous times (12th and 30th days p.i.) (Fig. 2). Interestingly, the pulsed- therapy with D-AMB was the better therapeutic regimen, in terms of controlling the fungal load in all organs, while the D-AMB monotherapy and the P-AMB pulse demonstrated a similar protective effect (Fig. 2).

The histopathological analysis confirmed the cfu counting results (Table 3). All therapeutic regimens reduced the fungal burden in the liver and kidneys compared to the untreated group on the 12th day p.i., with a greater reduction in the groups treated with D-AMB and D-AMB pulse (Table 3). The neutrophil reaction (NR) and the mononuclear phagocytic reaction (MPR) increased in this phase of infection in all groups, primarily in the liver. On the 45th day p.i., only the D-AMB pulse therapy was able to control the fungal burden in this assessment, but the leukocyte reaction remained sharp. Moreover, the analysis did not indicate the presence of fungi or leukocyte reaction in the kidneys of the animals treated with the D-AMB pulse regimen. In contrast, both parameters were observed in the mice treated with P-AMB pulse (Table 3).

Discussion

Although the therapeutic approach for sporotrichosis caused by *S. brasiliensis* has been performed according to

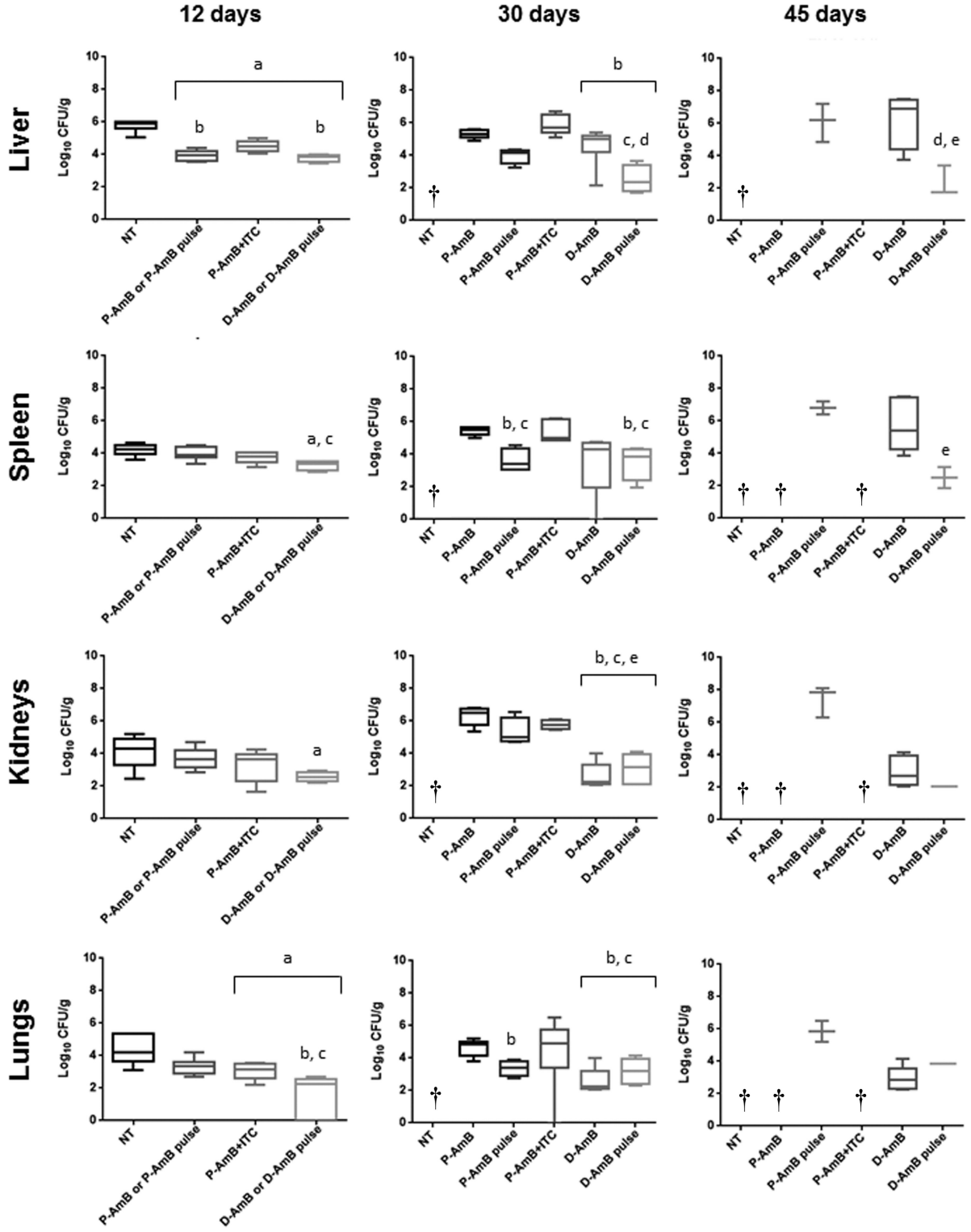

Figure 2. Determination of the fungal load in the selected organs (liver, kidneys, spleen, and lungs) of BALB/c male mice infected with yeasts of *Sporothrix brasiliensis* and treated with the following antifungal therapies: (a) monotherapy with 1 mg/kg/day of amphotericin B deoxycho-late (D-AMB) or 3 mg/kg/day of poly-aggregated amphotericin B (P-AMB), intravenously, for 7 days; (b) pulsed-therapy with D-AMB 1 mg/kg/day or P-AMB 3 mg/kg/day administered intravenously for 7 consecutive days, followed by a 7-day interval, and a new round of drug administration for 7 consecutive days using the same dose (D-AMB pulse and P-AMB pulse); (c) 3 mg/kg/day of P-AMB for 7 days followed by 75 mg/kg/day of itraconazole (37.5 mg/kg b.i.d. orally) up to 30 days post-infection (p.i.) (P-AMB/ITC). The fungal load was measured as the number of colony forming units per gram of tissue (cfu/g), and it was determined after 12, 30, and 45 days p.i. $n = 6$ mice per untreated (NT) and treated group. a, compared to the NT group ($P < .05$); b, compared to P-AMB/ITC ($P < .05$); c, compared to P-AMB ($P < .05$); d, compared to D-AMB ($P < .05$); e, compared to P-AMB pulse ($P < .05$); †, no surviving animals.

Table 3. Histopathological analysis of liver and kidneys of untreated BALB/c male mice infected with *S. brasiliensis* yeasts and treated with D-AMB or P-AMB monotherapy; poly-aggregated amphotericin B followed by itraconazole (P-AMB/ITC); or pulsed-therapy with D-AMB or P-AMB (D-AMB pulse and P-AMB pulse, respectively).

	Grades of the fungal burden and leucocyte reactions after antifungal therapy					
	Liver			Kidneys		
Antifungal treatments #	Fungal Burden**	NI**	MPI**	Fungal burden	NI	MPI
12th p.i.						
NT*	2–4	1	3–4	1–3	0–1	1
D-AMB	0	1–2	1–3	0–1	0	0–1
P-AMB	3	3	3	2	2	2
P-AMB /ITC	1	2	4	0	0	0
D-AMB pulse	0	1–2	1–3	0–1	0	0–1
P-AMB pulse	3	3	3	2	2	2
45th p.i.						
D-AMB	0–2	1–3	1–4	0	0	0
D-AMB pulse	0	1	0–2	0	0	0
P-AMB pulse	1–3	2	2–3	2–4	0–2	1–2

#For details of each therapeutic regimen see Material and Methods.

*NT, untreated group.

**Semiquantitative estimation of the fungal burden and leukocyte reactions in the tissues. NI, neutrophils infiltrate; MPI, mononuclear phagocyte infiltrate. For fungal burden: 0, none; 1, limited; 2, average; 3, high; 4, very high. For NI: 0 = none, 1 = some without abscesses, 2 = small abscesses' and 3 = large abscesses. For MPI: 0 = none, 1 = without granulomas, 2 = poorly formed granulomas, 3 = well-formed simple granulomas, and 4 = well-formed complex granulomas.

the specific *guidelines* for this disease,[11] the high toxicity of D-AMB and apparent lack of efficacy of ITC monotherapy motivated many studies to test the effect of new drugs to treat sporotrichosis or to improve the treatment protocols with recommended antifungal drugs.[4,5,9,31,32] Moreover, other antifungals as the new triazole agents, voriconazole and posaconazole, have demonstrated antifungal efficacy in a murine model of sporotrichosis,[14,15] and in disseminated sporotrichosis caused by *S. brasiliensis* in a patient with advanced AIDS.[33] Within this perspective, our study aimed to test (*in vitro* and *in vivo*) a new formulation of AMB, a poly-aggregated form (P-AMB), which has already demonstrated efficacy in murine models of leishmaniasis and candidiasis and also lowered toxicity compared to D-AMB.[22,24–26]

First, the *in vitro* antifungal susceptibility was verified for P-AMB towards *Sporothrix* spp. strains compared with D-AMB and ITC. In our study, the MIC data for D-AMB and ITC against filamentous and yeast forms were similar to previously published studies by other investigators.[10,12,13,34] The susceptibility testing showed that yeast, the pathogenic form of *Sporothrix* spp., had a lower susceptibility to P-AMB compared to D-AMB. These results led to adjustments in the doses of both D-AMB and P-AMB for the *in vivo* experiments.

P-AMB formulation showed lower cytotoxicity on LLC-MK_2 and RBC (2-4 times) compared to the D-AMB. Our *in vitro* results can be also supported by *in vivo* assays of the acute toxicity.[25–26,35] The lethal dose of 50% (LD_{50}) values for D-AMB and P-AMB in mice were 2–5 mg/kg and > 40 mg/kg, respectively (P-AMB less toxic at least 8 times than D-AMB).[25–26,35] Similar results were also observed for the liposomal amphotericin B formulation, which showed a maximum tolerated dose in mice approximately fivefold greater than D-AMB.[36] These *in vitro* and *in vivo* data may indicate that P-AMB is a therapeutic alternative in cases in which D-AMB cannot be recommended.[16] It is known that the dose and administration period for therapeutic use of D-AMB are limited due to renal toxicity; the drug causes irreversible renal injury in over 50% of patients.[37]

The *in vivo* experiments showed that the D-AMB monotherapy and pulsed-therapy with D-AMB (1 mg/kg) or P-AMB (3 mg/kg) was more effective in controlling the systemic infection by *S. brasiliensis* yeasts in BALB/c mice compared to the P-AMB monotherapy or the treatment with P-AMB followed by ITC. Recently, our group showed that administering 1 mg/kg/day D-AMB for 7 days followed by ITC (37.5 mg/kg b.i.d. orally) for up to 30 days p.i. was considered to be the most effective treatment in the same murine model of disseminated infection caused by *S. brasiliensis* compared with ITC or D-AMB monotherapies.[14] We also found that D-AMB monotherapy or D-AMB pulsed-therapy was more effective than P-AMB pulsed-therapy, despite the fact that the three therapeutic regimens cannot cure systemic sporotrichosis.

The results obtained in our work could be explained by the poly-aggregated molecular arrangement of the P-AMB formulation that could influence its antifungal efficacy. Different formulations of AMB have been tested in

some animal models of infection, demonstrating that the different molecular arrangements of AMB, in solution, can influence its effectiveness.[38–40] The aggregation of AMB molecules or their imprisonment in free lipid vesicles can control the release of free AMB, but a higher concentration of these formulations may be necessary to provide the same effectiveness of the D-AMB, which are organized in micelles.[38–40] Therefore, the chemical characteristics displayed by the P-AMB formulation were definitively reflected in the antifungal susceptibility assay, cytotoxicity, and efficacy of disseminated sporotrichosis treatment observed in the present work, as well as in the acute toxicity data previously showed by Espada et al.[25,26] Hence, although the P-AMB selective indexes towards *Sporothrix* spp. yeasts were similar or slightly less than D-AMB, the P-AMB formulation displays an important pharmacological characteristic—controlled drug release—which could minimize some acute effects during intravenous administration of D-AMB frequently reported in humans[41] and also observed in our work (e.g., the convulsion, followed by death, observed in the animals treated with D-AMB).

Histopathological analyses indicate that, despite the lower fungal burden in the liver, the mononuclear phagocyte response (RMF) in this organ remained high. These results are consistent with data in the literature concerning the immune response in murine sporotrichosis with effective participation of macrophages and monocytes in the mononuclear phagocyte system to combat *Sporothrix* infection. These cells play key roles in the innate immune response toward sporotrichosis.[42] In addition, the presence of P-AMB in the tissues may also induce an inflammatory reaction due to the capture of large molecular aggregates of AMB by the mononuclear phagocyte system in these organs.[43] It is known that different formulations of AMB (such as D-AMB and L-AMB) may stimulate the immune system inducing the proinflammatory cytokines production. The immunomodulatory properties of AMB can offer an alternative antifungal effect by enhancing the immune response of the host and contributed to the fungal elimination.[41]

In summary, our data reveal that D-AMB monotherapy and pulsed-therapy with D-AMB or P-AMB administered intravenously were the best therapeutic regimens to control the systemic murine sporotrichosis caused by the emerging species *S. brasiliensis*. In addition, P-AMB had a lower cytotoxic effect on renal cells and erythrocytes compared to D-AMB; and absence of acute symptoms after intravenous administration. Therefore, the polyaggregated AMB formulation might be an alternative to the sporotrichosis treatment, and our work may lead to the evaluation of other P-AMB therapeutic schemes for this mycosis.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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