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Original Article

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Amphotericin B, alone or followed by itraconazole therapy, is effective in the control of experimental disseminated sporotrichosis by *Sporothrix brasiliensis*

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Abstract

Sporothrix brasiliensis is a highly virulent member of the *S. schenckii* complex, which is responsible for the emergence of the epidemic sporotrichosis in southeastern Brazil over the last two decades. There are no *in vivo* studies on the sensitivity of *S. brasiliensis* to the therapeutic regimens used to treat sporotrichosis. Here, we evaluated the efficacy and safety of antifungal treatments against *S. brasiliensis* using a murine model of disseminated sporotrichosis. *In vitro*, *S. brasiliensis* yeasts were sensitive to low concentrations of amphotericin B–deoxycholate (AMB–d) and itraconazole (ITZ), the latter having greater selectivity toward the fungus. The following treatment regimens were tested *in vivo*: intravenous AMB–d for 7 days post-infection (p.i.), oral ITZ for up to 30 days p.i., and AMB–d followed by ITZ (AMB–d/ITZ). AMB–d and AMB–d/ITZ led to 100% survival of infected mice at the end of the 45-day experimental period. Although all treatments extended mice survival, only AMB–d and AMB–d/ITZ significantly reduced fungal load in all organs, but AMB–d/ITZ led to a more consistent decrease in overall fungal burden. No treatment increased the levels of serum toxicity biomarkers. Taken together, our results indicate that AMB–d/ITZ is the best therapeutic option for controlling disseminated sporotrichosis caused by *S. brasiliensis*.

Key words: amphotericin B, itraconazole, murine model, *Sporothrix brasiliensis*, sporotrichosis.

Introduction

Sporotrichosis is a subacute or chronic infection caused by thermodimorphic species of the *Sporothrix schenckii* complex, including *S. brasiliensis* [1,2]. This mycosis is becoming a public health problem in the state of Rio de Janeiro, Brazil, due a significant increase in human and animal cases over the last two decades. The leading source of infection in a recent outbreak of sporotrichosis is domestic cats, which can contribute to disease transmission to other animals and to humans by promoting direct inoculation of yeast cells into host tissues [3]. The primary species responsible for feline sporotrichosis in Brazil is *S. brasiliensis* [4]. Moreover, an epidemiological analyses showed that feline and human sporotrichosis caused by this fungus are spreading from Rio de Janeiro to neighboring Brazilian states, including São Paulo, Minas Gerais, and Paraná. The strains recovered in these states share the genotype found in the Rio de Janeiro outbreak [4]. Importantly, *S. brasiliensis* was identified in clinical specimens from the most severe cases of sporotrichosis reported in Brazil in recent years [5,6].

Sporotrichosis treatment depends on the type of clinical manifestation [7]. For cutaneous or lymphocutaneous disease, oral antifungal agents are the preferred treatment option, and the "gold standard" is one daily 200-mg dose of itraconazole (ITZ). Refractory disease is then treated with two daily oral doses of either ITZ (200 mg) or terbinafine (500 mg) or with three daily doses of a saturated solution of potassium iodide. For patients with life-threatening sporotrichosis (visceral or disseminated), treatment with intravenous amphotericin B (AMB) is required. AMB treatment may be followed by "maintenance" therapy with ITZ; this is the best option for controlling sporotrichosis disseminated in humans [7].

Different animal models, including subcutaneous and intravenous infection in mice, have been used to study the virulence of the two main pathogenic species, *S. schenckii sensu stricto* and *S. brasiliensis*; however, these models usually used the mycelial forms and conidia cells to promote the fungal infection [8–13]. There are no *in vivo* studies on the sensitivity of the yeast form of *S. brasiliensis* to the therapeutic regimens used to treat sporotrichosis. Actually, most of the Rio de Janeiro patients are contaminated directly by the yeast form of *S. brasiliensis* present in cats. Our aim was to evaluate the efficacy and safety of three therapeutic regimens for the treatment of sporotrichosis caused by yeasts from *S. brasiliensis* using a disseminated murine model of this fungal disease.

Materials and methods

Fungal strain

The highly virulent isolate of feline *S. brasiliensis* American Type Culture Collection (ATCC) MYA-4823 [9] used in this study was maintained in the mycelial form on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) at 28°C. For conversion to the yeast form, mycelia were subcultured twice in brain–heart infusion broth (Becton, Dickinson and Company, Sparks, MD, USA) at 36°C under orbital agitation.

Mice

Eight to 10-week-old male BALB/c mice weighing approximately 25 g were maintained in accordance with the National Institutes of Health Animal Care guidelines [14]. All procedures including euthanasia by carbon dioxide poisoning were approved by the Ethics Committee of the Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado Rio de Janeiro, Brazil (process number CEA/027/2010).

Antifungal drugs

For *in vitro* assays, ITZ (Sporanox; Janssen-Cilag Pharmaceuticals Inc., Sidney, New South Wales, Australia) and AMB– deoxycholate (AMB–d; Cristalia, São Paulo, Brazil) were diluted in dimethyl sulfoxide. For *in vivo* assays, ITZ and AMB–d were diluted in sterile phosphate-buffered saline (PBS) or 5% dextrose, respectively.

In vitro antifungal susceptibility assays

In vitro fungal susceptibility to drugs was assayed following Clinical Laboratory Standard Institute broth microdilution methods for mycelia [15] and yeast [16]. Fungal forms were treated with 0.03–16 μg/ml of AMB–d or ITZ for 5 days at 35°C. Wells with untreated fungal cells or only culture medium were used as positive and negative growth controls, respectively. The minimum inhibitory concentration (MIC) of antifungals was defined as the lowest concentration that inhibited more than 100% of fungal growth for AMB and ITZ.

Antifungal selective index toward *S. brasiliensis*

To evaluate the selectivity of AMB–d or ITZ toward *S. brasiliensis*, the cytotoxicity of these drugs to mammalian cells was estimated by treatment of monkey renal

cells (LLC-MK_2) and human red blood cells (RBCs) with 1–100 μg/ml of AMB–d or ITZ, as described previously [17]. CC_{50} (drug concentration yielding 50% cytotoxity to LLC-MK_2) and HA_{50} (drug concentration yielding 50% hemolytic activity) values were determined as described by Ishida et al. [17] and used to calculate drug selectivity indexes (SIs), according to the formula: SIw = CC_{50} or HA_{50}/yeast or mycelial MIC.

In vivo antifungal treatments and survival analysis

We used a modified version of the experimental model of disseminated murine sporotrichosis described by Teixeira et al. [11]. Briefly, mice were inoculated intravenously (through the lateral tail vein) with a suspension of 1×10^5 *S. brasiliensis* yeasts in PBS. Inoculum viability was verified by plating a volume of the yeast cell suspensions on PDA medium immediately after inoculation and determining the number of colony forming units (CFUs) after 7 days of incubation at 36°C.

Treatment of infected mice started 3 days post-infection (p.i.) with *S. brasiliensis* yeasts. Mice were divided into the following treatment groups: (a) 1 mg/kg/d AMB–d, administered intravenously for 7 days (AMB–d group); (b) 75 mg/kg/d ITZ, given orally by gavage (37.5 mg/kg twice daily) for up to 30 days p.i. (ITZ group); (c) treatment "a" followed by treatment "b" (AMB–d/ITZ group); or (d) control untreated mice. In addition, to analyze the effectiveness of early ITZ treatment, a group of mice was given ITZ monotherapy (as in "b") starting only 6 hours p.i. (eITZ group), simulating preventive therapy used when patients are scratched by infected cats, the most common form of contamination in Rio de Janeiro. Treated and untreated mice ($n = 9$ for each group) were monitored daily to evaluate the survival curve for at least 45 days p.i., and other parameters such as animal weight, physical condition, and behavior were also monitored.

Histopathological analysis and fungal load estimation

To analyze treatment effects on different organs, mice from all treatment groups were euthanized 3, 12, 30, or 45 days p.i. with *S. brasiliensis* ($n = 6$ mice for each group/time), and their kidneys, liver, lungs, and spleen were removed aseptically and weighed. Liver and kidney samples were fixed in 10% formaldehyde in PBS and prepared for histopathological examination. Paraffin sections of embedded organs were stained using the hematoxylin–eosin stain [18] and analyzed using a single-blind method of semiquantitative estimation of fungal burden and leukocyte reactions, according to the following scale: 0, none; 1, limited; 2, average; 3, high; 4, very high [19] (see also Supplementary Fig. 1).

To estimate fungal load in affected organs, 100 µl of a homogenate sample of each organ (kidneys, liver, lungs, and spleen) in PBS was plated onto PDA plates with 50 µg/ml chloramphenicol (Sigma Chemical Co., USA) and incubated at 28°C for 7 days. After incubation, colonies were counted to determine the colony-forming units per gram of tissue.

Analysis of serum toxicity biomarkers

To estimate treatment toxicity, blood was collected from anesthetized uninfected mice by cardiac puncture 12 or 30 days after the start of antifungal treatment from the following groups: AMB–d group (4 mice); ITZ group (4 mice); AMB–d/ITZ group (4 mice); and control untreated group (15 mice). The following four serum biomarkers of renal and hepatic toxicity were analyzed using kits from Labtest Diagnostica S.A. (São Paulo, Brazil): urea level, creatinine level, aspartate aminotransferase (AST) enzyme activity, and alanine aminotransferase (ALT) enzyme activity.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6.0 (Graph Pad Software Inc., USA), Student *t* test, and one-way analysis of variance. A P value ≤ 0.05 was considered statistically significant.

Results

Sporothrix brasiliensis yeasts are susceptible to low concentrations of AMB–d and ITZ *in vitro*

The antifungal activity of AMB–d and ITZ *in vitro* was evaluated against the highly virulent *S. brasiliensis* strain in its mycelia and yeast forms. While both *S. brasiliensis* fungal forms were equally susceptible to AMB–d (MIC = 0.5 µg/ml), mycelia were much less sensitive to ITZ (MIC = 4 µg/ml) than the yeast form (MIC = 0.5 µg/ml; Table 1). The cytotoxic and hemolytic activities of ITZ (against LLC-MK_2 cells and human RBC, respectively) were considerably lower than those observed for AMB–d, resulting in higher SI yeast values for ITZ, which was 8-fold more selective to yeast than to mycelia (Table 1).

Treatment with AMB–d, alone or followed by ITZ, led to 100% survival of mice infected with *S. brasiliensis*

A murine model of disseminated sporotrichosis was used to estimate the efficacy of ITZ and AMB–d against

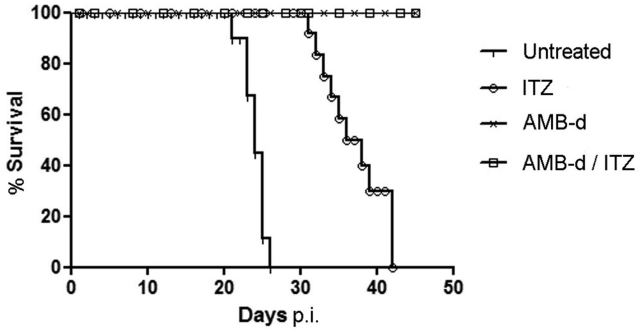

Figure 1. Survival rate of BALB/c mice infected with *Sporothrix brasiliensis* (American Type Culture Collection MYA-4823) yeasts and subjected to one of the following treatments: (a) 1 mg/kg/d amphotericin B–deoxycholate (intravenously) for 7 days (AMB–d group); (b) 75 mg/kg/d itraconazole (37.5 mg/kg twice daily orally) for 30 days (ITZ group); or (c) treatment "a" followed by treatment "b" (AMB–d/ITZ group). Antifungal treatments started 3 days after yeast inoculation. Infected but untreated mice were used as the control group. $n = 9$ mice per treatment group; p.i., post-infection.

infections by highly virulent *S. brasiliensis* yeasts (ATCC MYA-4823). Untreated animals infected with *S. brasiliensis* reached 100% mortality 26 days p.i. (Fig. 1). While all therapeutic schemes prolonged the survival of infected mice ($P < 0.0001$), treatment with AMB–d alone or followed by ITZ showed the highest efficacy, leading to a survival rate of 100% 45 days p.i. In contrast, no mice treated with ITZ alone survived for more than 42 days p.i. (Fig. 1). All infected animals experienced weight loss and were listless, with shallow breathing and reduced food and water intake; however, the untreated group showed prominent effects as early as 12 days p.i.

AMB–d and AMB–d/ITZ treatments reduced fungal load in affected organs of mice infected with *S. brasiliensis*

The fungal load in selected organs of mice infected with *S. brasiliensis* ATCC MYA-4823 was estimated as the number of fungal colonies per gram of tissue (Fig. 2). Untreated mice displayed a significant increase in fungal load in the liver (40%; $P = 0.001$) and kidneys (25%; $P = 0.02$) but not in the spleen and lungs 12 days p.i. compared with pretreatment controls (Fig. 2). In contrast, both AMB–d alone and AMB–d followed by ITZ led to a significant reduction (30%–40%) in fungal load in all organs analyzed in comparison with the untreated group (Fig. 2). No statistically significant differences were observed in fungal load between AMB–d and AMB–d/ITZ groups at any of the time points p.i. (12, 30, or 45 days), except for liver on day 30, where we observed a more reduced fungal load after treatment with AMB–d/ITZ than with AMB–d alone ($P < 0.001$). ITZ treatment was the least efficient at reducing fungal load (Fig. 2). At 12 days p.i., the fungal load in all examined organs of mice subjected to ITZ treatment was similar to that observed in untreated mice. However, fungal load had increased 30 days p.i. in the liver and kidneys (Fig. 2), and, as mentioned previously, no mice in the ITZ group survived 42 days p.i. (Fig. 1).

Interestingly, when ITZ treatment was initiated 6 hours (0.25 days) p.i., 100% mouse survival was achieved up to 42 days p.i. ($P < 0.0001$; Fig. 3A). Compared with 6 h p.i., untreated mice had a significant increase in fungal load in the liver only after 12 days p.i. (approximately 30%; $P = 0.001$; Fig. 3B–E). This increase was not observed in mice treated with ITZ from 6 h p.i. (Fig. 3B–E). Indeed, early ITZ treatment promoted a reduction (20%–30%) of fungal load in all organs, except the kidneys, 12 days p.i. when compared with the fungal load observed before treatment (Fig. 3B–E). Importantly, early treatment with ITZ was successful in controlling the fungal load in all studied organs up to 45 days p.i. (Fig. 3B–E).

AMB–d and AMB–d/ITZ promote a sustained reduction in fungal burden and leukocyte reactions in mice infected with *S. brasiliensis*

According to the histopathological analysis, all treated mice showed a reduction in the fungal burden in the liver and kidneys 12 days p.i. when compared with untreated mice (see Supplementary Materials, Supplementary Fig. 1, and Supplementary Table 1). In some tissue samples, fungal cells

Table 1. Antifungal susceptibility and selectivity of itraconazole and amphotericin B deoxycholate toward yeast and mycelial forms of *Sporothrix brasiliensis,* American Type Culture Collection strain MYA-4823.

Drug	MIC (μg/ml)		Cytotoxicity (μg/ml) and SI			Hemolytic activity (μg/ml) and SI		
	Mycelia	Yeast	CC_{50}	SImyc	SIyeast	HA_{50}	SImyc	SIyeast
ITZ	4	0.5	>100	>25	>200	>100	>25	>200
AMB-d	0.5	0.5	6.25	12.5	12.5	17	34	34

Selectivity indexes were calculated using the formula: SI = CC_{50} or HA_{50}/MIC.
CC_{50}, drug concentration yielding 50% cytotoxicity to monkey renal cells (LLC-MK_2); HA_{50}, drug concentration yielding 50% hemolytic activity to human red blood cells; MIC, minimum inhibitory concentration; SI, selectivity index; SImyc, SI for mycelia; SIyeast, SI for yeasts.

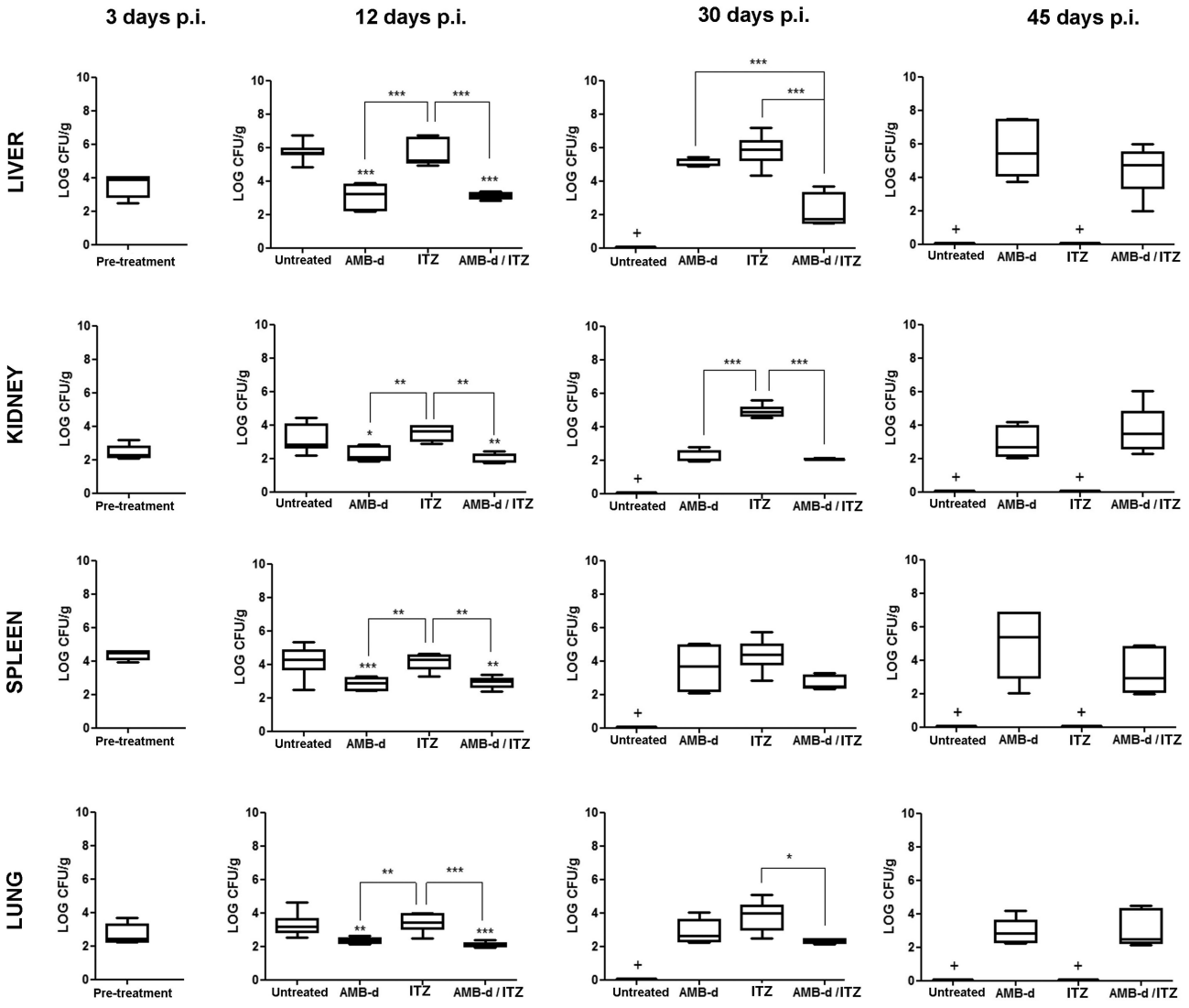

Figure 2. Evaluation of fungal load in selected organs (liver, kidneys, spleen, and lungs) of BALB/c mice infected with *Sporothrix brasiliensis* (American Type Culture Collection MYA-4823) yeasts and subjected to one of the following treatments: (a) 1 mg/kg/d amphotericin B deoxycholate (intravenously) for 7 days (AMB–d group); (b) 75 mg/kg/d itraconazole (37.5 mg/kg twice daily orally) for 30 days (ITZ group); or (c) treatment "a" followed by treatment "b" (AMB–d/ITZ group). Pretreated and untreated groups were included in this study. Fungal load was measured as the number of colony forming units per gram of tissue (CFU/g). Treatment started 3 days post-infection (p.i.) with *S. brasiliensis* yeasts, and fungal load was determined after 3, 12, 30, and 45 days p.i. $^{*}P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. +, no surviving animals.

could be found in inflamed or abscessed regions in the liver (all antifungal treatments) or kidneys (ITZ or AMB–d/ITZ treatments; data not show). In contrast, untreated animals displayed more drastic liver injury, with areas of necrosis (data not shown). Intense mononuclear phagocyte system reactions (MPSRs) and modest neutrophilic reactions (NRs) were observed in the liver of mice from all treated groups 12 days p.i., while leukocyte reactions in the kidneys were less pronounced (Supplementary Table 1).

The fungal burden in the liver and kidneys was reduced in all mice surviving 45 days p.i. in the AMB–d, AMB–d/ITZ, and eITZ groups (Supplementary Table 1), and yeast cells could be found inside macrophages or in regions of kidney or liver abscess or necrosis in mice from the AMB–d and AMB–d/ITZ groups (data not shown). NRs of similar intensity were observed in all organs, while MPSRs were more pronounced in some samples, especially in the liver (Supplementary Table 1).

Serum toxicity biomarker levels are not affected by AMB–d and ITZ treatments

We measured the serum biomarker levels of liver and renal toxicity in treated but uninfected mice to determine if any of the treatments used elicited appreciable toxic effects. No significant alterations in toxicity biomarkers (urea and

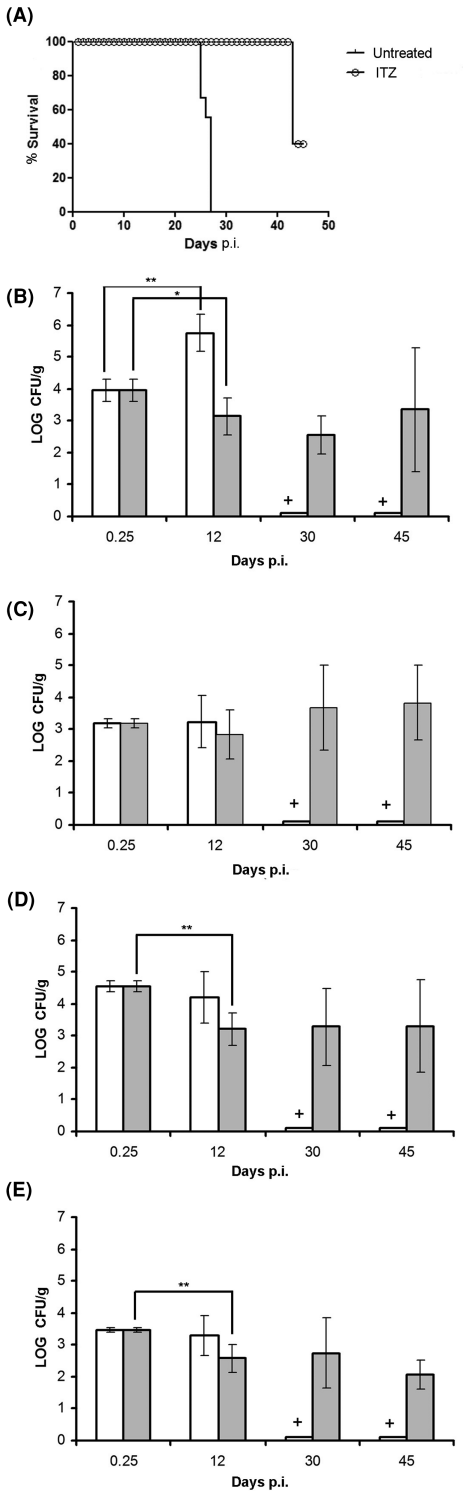

Figure 3. Survival rate (A) and evaluation of fungal load in the liver (B), kidneys (C), spleen (D), and lungs (E) of BALB/c mice infected with *Sporothrix brasiliensis* American Type Culture Collection MYA-4823 yeasts and treated with itraconazole 75 mg/kg/d (37.5 mg/kg twice daily orally) starting from 6 hours post-infection (p.i.) for 30 days (eITZ group). Fungal load was determined before treatment (0.25 day p.i.) and 3, 12, 30, and 45 days p.i. as the number of colony forming units per gram of tissue (CFU/g). Infected untreated mice were used as a control group. White bars, untreated mice; gray bars, ITZ treatment. $^{*}P < 0.05$; $^{**} < 0.01$. +, no surviving animals.

creatinine levels and AST and ALT enzymatic activities) were detected after 12 or 30 days of antifungal therapy in any of the treatment groups when compared with untreated animals (see Supplementary Table 2).

Discussion

In this study, we evaluated the efficacy and safety of ITZ and AMB–d administration, alone or as sequential treatments, against sporotrichosis caused by a highly virulent *S. brasiliensis* strain, in an *in vivo* model of disseminated disease. We are the first to evaluate antifungal therapy with AMB–d and ITZ as maintenance treatment in a murine model of disseminated sporotrichosis caused by yeast cells of *S. brasiliensis*. We chose the ATCC MYA-4823 isolate of *S. brasiliensis* for this study because its high virulence had been demonstrated in murine models of sporotrichosis [9,13].

Although similar MIC values were obtained *in vitro* for *S. brasiliensis* yeasts with AMB–d or ITZ (0.5 mg/ml), treatment with AMB–d, alone or followed by ITZ, was the most effective therapy *in vivo* against disseminated sporotrichosis by *S. brasiliensis*. These results are similar to those described by Kan and Bennett [20], on the antifungal treatment of disseminated murine sporotrichosis by *S. schenckii* isolates. Those authors reported that AMB–d was the most effective treatment against disseminated sporotrichosis, resulting in 100% survival 35 days p.i., with the fewest visible lesions and fungal burden in internal organs [20]. As in our study, Kan and Bennett [20] also observed that ITZ treatment increased mouse survival (90% up to 35 days) but did not fully protect the animals from disseminated disease in the brain, bones, and viscera. Although we observed prolonged survival after ITZ treatment (approximately 80% 35 day p.i.), all animals succumbed by 45 days p.i. (Fig. 1).

Serum biomarkers of renal and hepatic toxicity (urea and creatinine levels and AST and ALT enzymatic activities) were evaluated in noninfected mice subjected to up to 30 days of antifungal treatments. No alterations were observed in serum of these animals, indicating that all therapy regimens could be used safely, despite the fact that AMB–d had low SI values *in vitro*, suggestive of toxicity to mammalian cells.

Although both AMB–d and AMB–d/ITZ treatments led to 100% survival of infected mice at the end of the experimental period, we observed less variability in the fungal load among mice treated with AMB–d/ITZ compared with those treated with AMB–d alone (Fig. 2). Histopathological analysis also showed a reduction in the number of fungal cells in mice treated with AMB–d/ITZ, and inflammatory processes were prominent in this group

(Supplementary Table 1). Therefore, we describe the overall efficiency of the therapeutic schemes tested on the control of fungal load in mice with disseminated sporotrichosis as follows: AMB–d/ITZ > AMB–d > ITZ. Although ITZ is recommend as the first choice when treating cutaneous and lymphocutaneous forms of sporotrichosis [7], the results presented here show that this drug does not seem to efficiently treat severe cases caused by *S. brasiliensis*, confirming recommendations describe by Kauffman et al. 2007 [7]. However, when ITZ treatment was initialized 6 hours p.i. (eITZ), it prolonged mouse survival until 42 days p.i., indicating that earlier ITZ treatment is more efficient than late treatment, especially in cases of *S. brasiliensis* infection where treatment outcome is dependent on early diagnosis and treatment. These data indicate that ITZ can be considered for treating healthcare professionals and researchers who are accidentally infected by *S. brasiliensis* or individuals scratched by infected cats, which is the most usual form of contamination by *S. brasiliensis* in Rio de Janeiro.

We show that AMB–d can be used alone as the drug of choice for the treatment of disseminated *S. brasiliensis* infection; however, the best option is likely to be AMB–d followed by maintenance treatment with ITZ. These data are in agreement with study results from the limited number of clinical case studies available on the combined use of AMB–d and ITZ [21,22]. Kohler et al. [21] described a case of a diabetic patient with localized osteoarticular sporotrichosis followed by widespread dissemination of the infection, which was successfully treated with AMB–d followed by oral fluconazole as maintenance treatment. In addition to its use as maintenance therapy, oral ITZ was also successfully combined with intralesional AMB–d in the treatment of refractory feline sporotrichosis [22].

Conclusions

Our data show that AMB–d followed by ITZ was the best therapeutic regimen for controlling disseminated murine sporotrichosis caused by the emerging *S. brasiliensis* yeast. These findings are in agreement with the Infectious Diseases Society of America guidelines for treating disseminated sporotrichosis in humans, strongly suggesting that the current first-line therapy used for other species of the *S. schenckii* complex is also valid for *S. brasiliensis*. In addition, our work suggests that the murine model of *S. brasiliensis* sporotrichosis used here is likely to yield clinically meaningful results during *in vivo* testing of new prospective therapies against *S. brasiliensis*.

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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.

Supplementary Material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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