Efficacy of posaconazole in a murine model of systemic infection by *Saprochaete capitata*

Running title: Posaconazole against *Saprochaete capitata*

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Abstract

The fungus *Saprochaete capitata* causes opportunistic human infections, mainly in immunocompromised patients with haematological malignancies. The best therapy for this severe infection is still unknown. We evaluated the *in vitro* killing activity and the *in vivo* efficacy of posaconazole at 5, 10, or 20 mg/kg BID in a murine neutropenic model of systemic infection by *S. capitata* testing a set of six clinical isolates. Posaconazole showed fungistatic activity against all the isolates tested. The different doses of the drug, especially the highest one, showed good efficacy, measured by prolonging survival, reduction of (1-3)-β-D-glucan serum levels, tissue burden reduction and histopathology.

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INTRODUCTION

Saprochaete capitata, formerly known as *Trichosporom capitatum*, *Geotrichum capitatum* and *Blastoschizomyces capitatus* is an uncommon clinical fungus belonging to the Basidiomycota, but able to cause fatal fungemia in immunocompromised patients, especially in those with haematological malignancies (1-6). The therapeutic options against these infections are limited, *S. capitata* being considered intrinsically resistant to the echinocandins (7-10). Currently there are no recommendations for the management of infections caused by *S. capitata,* although amphotericin B is the drug most commonly used in the clinical setting, followed by itraconazole and voriconazole (9, 11-14). The use of these compounds is supported by the *in vitro* antifungal susceptibility of *S. capitata* to such drugs. However, despite treatment, mortality still remains high at around 60% (5, 15-19) making it necessary to explore new therapeutic approaches. In previous studies conducted on mice, high doses of fluconazole demonstrated higher efficacy than amphotericin B, flucytosine, and voriconazole (20). Posaconazole has not been evaluated against this fungal species before but has shown efficacy in experimental infections against a wide range of opportunistic fungi such as *Aspergillus* spp., *Curvularia* spp, *Rhizopus oryzae* (21, 22, 23) among others, including *Trichosporon asahii* which is taxonomically related to *S. capitata* (24). In the present study, we evaluated the *in vitro* and killing activity of posaconazole against this fungus as well as its *in vivo* efficacy in a neutropenic murine model of systemic infection by *S. capitata*.

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MATERIAL AND METHODS

Strains and inocula

Six clinical strains of *S. capitata* (IHEM 5665, IHEM 5666, IHEM 5091, IHEM 6803, IHEM 6105 and IHEM 16109) were included in the study. The inocula were prepared from potato dextrose agar (PDA) cultures by flooding the plates with 3 ml of sterile saline solution and scraping the surface of the colonies with a loop, in order to obtain a conidial suspension. To remove hyphal fragments and clumps of agar, the resulting suspension was filtered twice through sterile gauze and then adjusted by haemocytometer counts to the desired concentrations. Inocula viability was determined by placing 10-fold dilutions of the conidial suspension on PDA plates.

In vitro studies

Pure posaconazole powder provided by Schering-Plough (Kenilworth, NJ) was used in the *in vitro* study following the reference microdilution method according to the CLSI document M27-A3 (25). Time kill curves were performed as previously described (26). In brief, two-fold serial dilutions, ranging from 64 to 0.06 µg/ml of posaconazole were assayed. At predetermined time points (0, 4, 8, 24 and 48 h) aliquots of 100 µl were removed, serially diluted in sterile water, placed onto PDA plates and incubated at 35°C for 24-48 h in order to determine the CFU/ml. This procedure allowed a limit of detection of 33 CFU/ ml. All assays were carried out in duplicate and the geometric mean and standard deviation were calculated. A reduction on CFU counts of $\geq$ 99.9 % or 3 $\log_{10}$ compared to the starting inoculum was considered indicative of fungicidal activity, while a CFU count reduction of < 99. 9% was considered fungistatic (27).

In vivo studies

Four-week-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain), weighing 28–30 g were used. All animals included in the study were immunosuppressed by intraperitoneal administration of a single dose of 200 mg/kg of cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) 2 days prior to the infection and then every 5 days until the end of the experiment (28). In order to prevent bacterial infections all animals received 5 mg/kg/day of ceftazidime subcutaneously. Mice were inoculated intravenously (i.v.) with 2×10^6 CFU/animal of each fungal strain in 0.2 ml of sterile saline solution into the lateral tail vein. This inoculum has previously proven appropriate for producing an acute infection (20). Animals were housed under standard conditions, and care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare Committee. The efficacy of posaconazole was evaluated by prolongation of survival, (1-3)-β-D-glucan serum levels, reduction of tissue burden and histopathologic features.

Groups of thirteen mice were randomly established, 5 for survival and 8 for tissue burden and determination of (1→3)-β-D-glucan levels in serum samples.

In a preliminary study, animals were challenged with the strains IHEM 5666 and IHEM 16105 and the efficacy of posaconazole was assayed at increasing doses of 5, 10 and 20 mg/kg twice daily (BID) orally by gavage for 6 days starting 24h after infection to determine the most effective. The doses were selected from time-kill results and previous drug pharmacodynamics studies (29, 30). Since posaconazole at 10mg/kg already showed good efficacy this dose was chosen to be tested against the four remaining strains in a second study.

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107 Determination of glucan and drug levels and fungal load

108 Control and treated mice from the tissue burden study group, were anaesthetized by
109 inhalation of sevofluorane (Sevorane; Abbott, Madrid, Spain) on day 7 post infection and
110 12 h after the last dose was administered, 1 ml of blood from each mouse was extracted by
111 cardiac puncture. Animals were then euthanased by cervical dislocation. Serum samples
112 were obtained by centrifugation of the blood at 3500 rpm and were stored at -20 ° C until
113 their use. Serum levels of (1→3)-β-D-glucan were determined using the Fungitell kit
114 (Associates of Cape Cod, East Falmouth, MA, USA) following the manufacturer's
115 instructions and levels of drug by bioassay, as previously described (31). Liver, spleen,
116 lungs, kidneys and brain of animals were aseptically removed and approximately one half
117 of each organ was weighed and mechanically homogenized in 1 ml of sterile saline
118 solution. Homogenates were serially diluted (1:10), placed onto PDA plates and incubated
119 for 48 h at 35°C for fungal load calculation (CFU/g of tissue).

120 Histopathology

121 The other half of each organ was fixed with 10% buffered formaldehyde. Samples were
122 embedded in paraffin and stained with hematoxylin-eosin, periodic acid-Shiff and Grocott
123 methenamine silver and examined in blinded fashion by light microscopy.

124 Statistical analysis

The mean survival times were estimated by Kaplan-Meier method and compared among groups using the log rank test. Results from the tissue burden studies were analysed using the Mann-Whitney *U*-test, and the Kolmogorov-Smirnov test was carried out to determine the normal distribution of (1→3)-β-D-glucan serum levels by GraphPad Prism 6.0 for Microsoft Windows (GraphPad Software, San Diego California USA). A *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Posaconazole showed fungistatic activity against the six strains of *S. capitata* tested with a reduction in the viability of ≤ 0.14 log $_{10}$ CFU/ml. Figure 1 illustrates the time-killing kinetic assay against IHEM 16105 as representative of the all strains assayed. Additionally, the MIC value was 0.25 µg/ml against all of them.

The dose escalation study showed efficacy of posaconazole 10 and 20 mg/kg against the two strains tested in this first study in comparison to the control group ($P \leq 0.016$), while posaconazole 5 mg/kg BID showed efficacy against only one of the two strains (IHEM 16105) (P=0.029) (Fig. 2). The dose of 10 mg/kg BID was chosen for the second study for treating infections by the six strains, and prolonged significantly the survival with respect to the control group ($P \leq 0.048$) (Table 1).

The six strains tested caused high fungal load in all organs, the kidneys and brain generally being the most affected (mean log10 CFU/g tissue ≥ 7.41 and ≥ 7.31, respectively). For the strains IHEM 5666 and IHEM 16105 any dose of posaconazole reduced significantly the fungal load in comparison to the control in all organs studied ($P \leq 0.0079$) (Fig. 3), as well as posaconazole 10 mg/kg BID did against the rest of the strains ($P \leq 0.0079$) (Table 2).

Reduction of CFU/g in animals receiving posaconazole 10 respect to the control animals, was ranged from 1.83 to 3.42 $\log_{10}$ being the highest reduction observed in liver (mean $\log_{10}$ ± SD, 2.9 ± 0.41) and the lowest in brain (2.13 ± 0.21). Twelve hours after the end of the treatment with posaconazole 5, 10 and 20 mg/kg, serum levels of drug were (mean ± SD) 5.76 ± 0.5, 6.48 ± 0.75 and 7.46 ± 0.70 µg/ml, respectively being all above the MIC values. At day 7 post infection the (1→3)-β-D-glucan serum levels of the controls ranged from 360 to 503 pg/ml. posaconazole at 5, 10 or 20 mg/kg BID was able to reduce the (1→3)-β-D- glucan serum concentrations in comparison with the untreated group although not below the cut-off for positivity in human infections, which is 80 pg/ml (32) (Fig. 4).

The histopathologic studies also confirmed that kidney and brain were the most affected organs in untreated animals. Presence of necrotic and haemorrhagic foci with no inflammatory response and abundant fungal structures located in the parenchyma and associated to angioinvasion were observed in all the studied organs (Fig. 5). In mice receiving the different doses of posaconazole, the presence of fungal cells was reduced in a dose-dependent manner, being observed focally in the parenchyma with no sign of necrosis or angioinvasion

DISCUSSION

S. capitata causes serious opportunistic infections in patients with haematological malignancies, especially in those with acute leukaemia, with a poor outcome (2, 4, 5). Although an improvement in neutropenia in patients with systemic infections by *S. capitata* leads to better prognosis, it is not enough to cure the infection (2, 4-6).

In this study, we evaluated the efficacy of posaconazole against an unusual number of clinical strains for this type of study, trying to evaluate the possible intra-species variability in antifungal response, as there is in many species. Therefore, we selected six clinical strains of *S capitata* with identical MICs and similar time-kill kinetics of posaconazole. Other authors have also reported that posaconazole is active *in vitro* against *S. capitata* with MICs ranging from 0.016 to 1 µg/ml (3, 5, 9) although MICs ≥ 4 µg/ml have occasionally been reported (18).

In this study, posaconazole at any dose prolonged the survival of the animals compared to the control group, the best results being obtained with posaconazole 10 and 20 mg/kg BID. In addition, posaconazole at any dose significantly reduced fungal burden in all the studied organs as well as the (1→3)-β-D-glucan serum levels. Such reduction was dose dependant and correlates with the serum levels of drug detected after the end of the treatment. The (1→3)-β-D-glucan marker is a cell wall component common in the fungi kingdom, easily detectable and quantifiable in serum and body fluids, and is used as marker of disseminated fungal infections, including those by *S. capitata* (9, 33). We found a correlation between the decrease in (such antigen, the fungal load and the dose administered. Up to now, the detectable levels of (1→3)-β-D-glucan have been used for diagnosis although the relationship between such antigen levels and the fungal load found in the present study and in previous studies on animal models (9, 33) seems to indicate that (1→3)-β-D-glucan levels might be useful for evaluating prognosis in infections by *S. capitata.* Further studies are needed to confirm this finding.

As indicated above, the efficacy of other drugs such as amphotericin B, flucytosine, voriconazole and fluconazole were previously evaluated in a systemic infection by *S.*

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192 *capitata* using a murine model and showed that fluconazole at a high dose (80 mg/kg) was
193 the most effective in prolonging the survival of mice and reducing the fungal burden in
194 liver, spleen and kidney (20) similarly as posaconazole did in the present study. Despite
195 good results obtained with fluconazole in the treatment of the experimental infection by *S.*
196 *capitata*, an important limitation to its use is the reported resistance to that drug *in vitro* (1).
197 Other reports indicate the lack of susceptibility of *S. capitata* to fluconazole and/ or
198 echinocandin, given that these antifungal compounds are administered empirically to
199 prevent or treat infections by other fungal in patients with haematological malignancies (9).
200 Considering the little experience in the management of systemic infections by *S. capitata*
201 and the risk of acquired resistance to fluconazole and echinocandins, our results are of
202 special interest. posaconazole, which is a well-tolerated drug treatment could another useful
203 tool in our fight against this difficult-to-treat infection, especially when other therapeutic
204 options fail.

205

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Table 1. Survival of mice infected with 6 different *S. capitata* strains and treated with posaconazole 10 mg/kg BID (PSC 10). † MST, mean survival time; ‡ 95% CI, 95% confidence interval.

Strains	MST † (days) and [95% CI] ‡		*P* value
	Control	PSC 10	
IHEM 5665	9.2 [6.9 - 11.42]	17.8 [8.8 - 26.73]	0.0039
IHEM 5666	7.2 [5.58 - 8.81]	14.20[9.6 - 18.70]	0.0025
IHEM 5091	7.6 [5.71 - 9.48]	17 [7.8 - 26.17]	0.0039
IHEM 6803	9.4 [7.7 - 11.07]	15.40 [10.70 - 20.10]	0.0048
IHEM 16105	8.8 [7.18 - 10.42]	11.8 [8.46 - 15.13]	0.0027
IHEM 16109	8.6 [5.88-11.32]	13.4 [8.70-18.10]	0.0480

Table 2. Effects of antifungal treatment on colony counts in the liver, lung, kidney, brain and spleen of neutropenic mice infected with 2 x 10^6 CFU of S. *capitata*. Animals received treatment comprising posaconazole at 10 mg/kg BID (PSC 10) for 6 days. † 95% CI, 95% confidence interval; ‡ P value, P value in comparison to the control group.

		Log10 CFU/g of tissue (95% CI) † *P value* ‡				
Strain	**Treatment**	**Liver**	**Lung**	**Kidney**	**Brain**	**Spleen**
IHEM 5665	Control	6.31 (5.88-6.92)	6.76 (6.11-7.11)	7.88 (7.75-8.10)	8.01 (7.69-8.45)	6.17 (5.80-6.45)
	PSC 10	2.89 (2.48- 3.27) *0.0002*	3.69 (2.90-4.50) *0.0002*	5.52 (5.25-5.90) *0.0002*	6.00 (5.65-6.68) *0.0002*	3.69 (3.30- 4.17) *0.007*
IHEM 5666	Control	6.24 (5.78-6.82)	6.35 (5.95-6.94)	8.00 (7.44-8.93)	7.51 (6.98-8.01)	6.23 (5.45-6.86)
	PSC 10	3.85 (3.27-4.57) *0.0070*	4.04 (3.49-4.70) *0.0002*	5.75 (5.00-6.35) *0.0002*	5.48 (4.45-6.62) *0.0002*	3.84 (3.1-4.75) *0.0002*
IHEM 5091	Control	6.11 (5.25-7.28)	6.12 (5.58-6.62)	7.68 (7.34-8.00)	7.31 (6.62-7.81)	6.04 (5.60-6.40)
	PSC 10	2.89 (2.20-3.42) *0.0002*	2.97 (2.41-3.30) *0.0002*	5.07 (4.00-5.88) *0.0002*	5.20 (4.10-5.92) *0.0002*	3.61 (3.00-4.15) *0.0002*
IHEM 6803	Control	6.35 (5.80-6.73)	6.68 (6.10-7.10)	8.07 (7.78-8.50)	7.54 (7.20-7.82)	6.47 (5.57- 7.74)
	PSC 10	3.31 (3.10-3.61) *0.0002*	3.43 (2.80-4.25) *0.0002*	5.35 (4.9-5.96) *0.0002*	5.55 (4.90-5.96) *0.0002*	3.23 (2.90-3.39) *0.0002*
IHEM 16105	Control	6.71 (5.97-7.67)	6.68 (6.19-7.26)	7.78 (7.53-8.17)	7.64 (6.99-8.17)	6.85 (6.64-7.18)
	PSC 10	4.24 (4.05-4.39) *0.0002*	4.85 (4.42-5.41) *0.0002*	5.37 (4.71-5.80) *0.0002*	5.08 (4.30-5.64) *0.0002*	4.57 (4.29-5.10) *0.0002*
IHEM 16109	Control	6.09 (5.88-6.26)	6.29 (5.12-6.99)	7.41 (6.98-7.86)	7.51 (6.70-8.01)	6.98 (6.3-6.46)
	PSC 10	3.21 (2.30-4.07) *0.0002*	3.53 (2.80-4.37) *0.0002*	5.29 (4.62-5.75) *0.0002*	5.41 (5.02-5.83) *0.0002*	3.65 (3.10-4.32) *0.0002*

Figure 1. Time-killing kinetic assay of PSC against IHEM 16105 *S. capitata* strain.

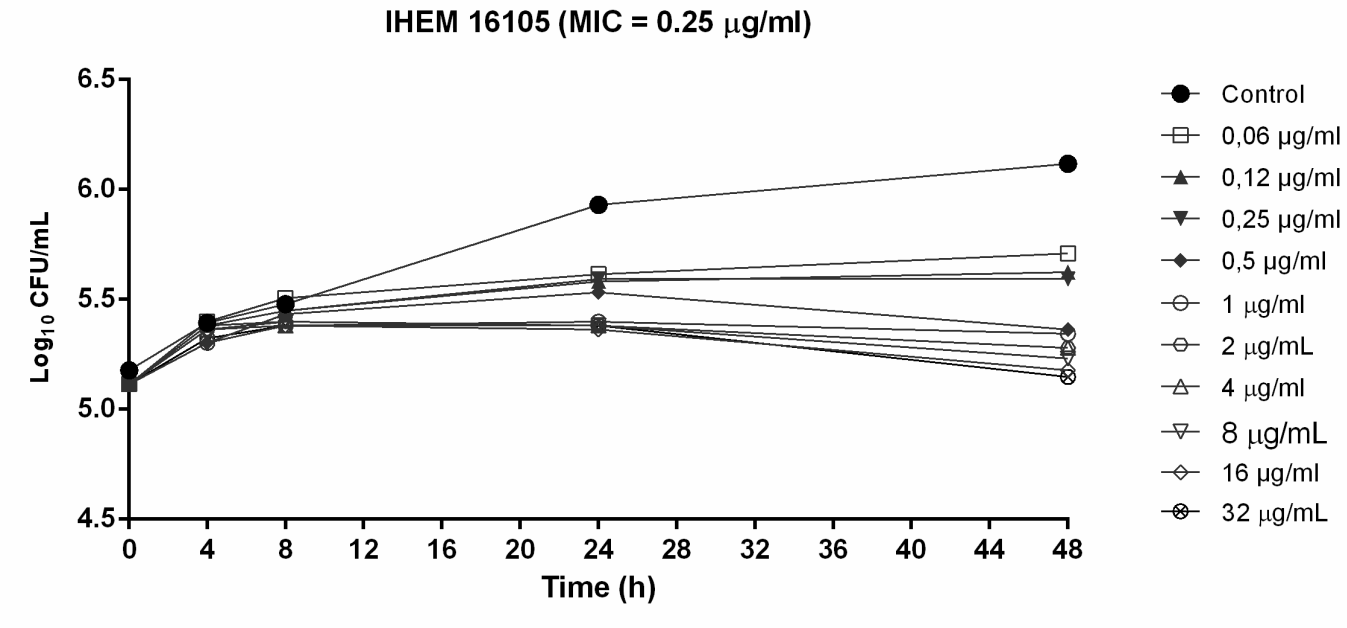
Figure 2. Survival of neutropenic mice infected intravenously with 2 x 10^6 colony-forming units of *S. capitata* IHEM 5666 and IHEM 16105. Animals were treated for 6 days with posaconazole (PSC) at 5 mg/kg BID; 10 mg/kg BID or 20 mg/kg BID. [a] $P \leq 0.02$ versus control, [b] $P = 0.008$ versus PSC 5.

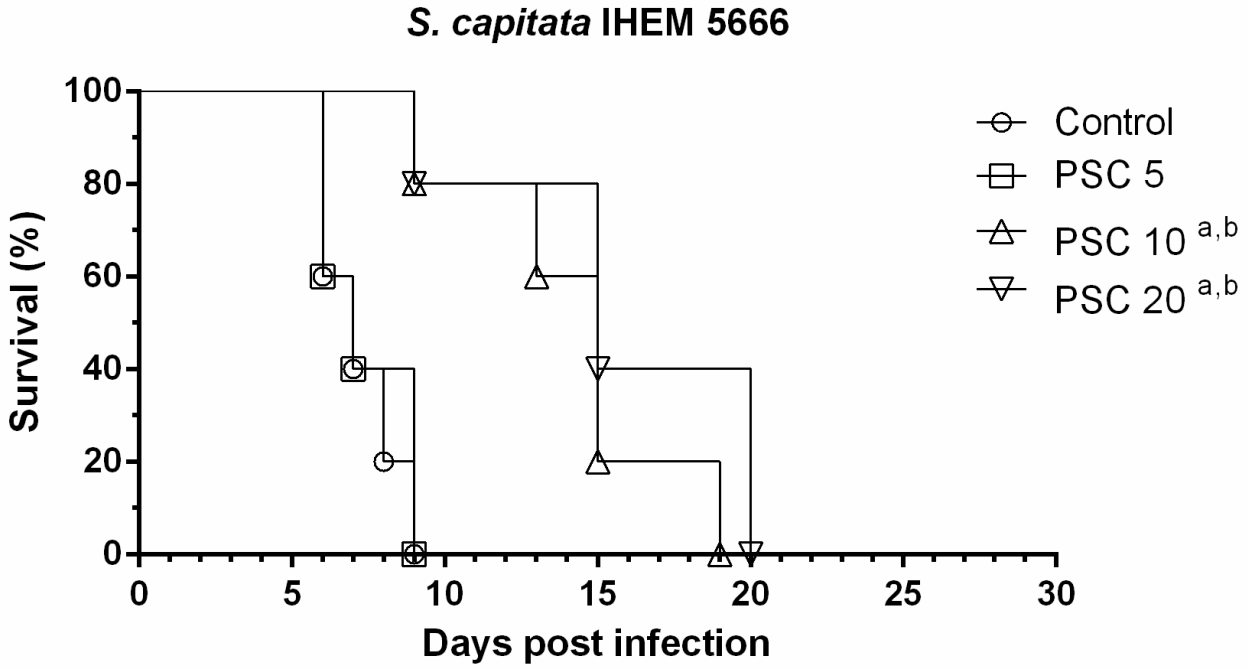
Figure 3. Effects of antifungal treatment on colony counts of neutropenic mice infected with 2 x 10 [6] CFU of *S. capitata* in liver, lung, kidney, brain and spleen and treated for 6 days with posaconazole (PSC) at 5 mg/kg BID; 10 mg/kg BID or 20 mg/kg BID. [a] $P \leq 0.007$ versus control; [b] $P \leq 0.007$ versus PSC 5; [c] $P \leq 0.014$ versus PSC 10.

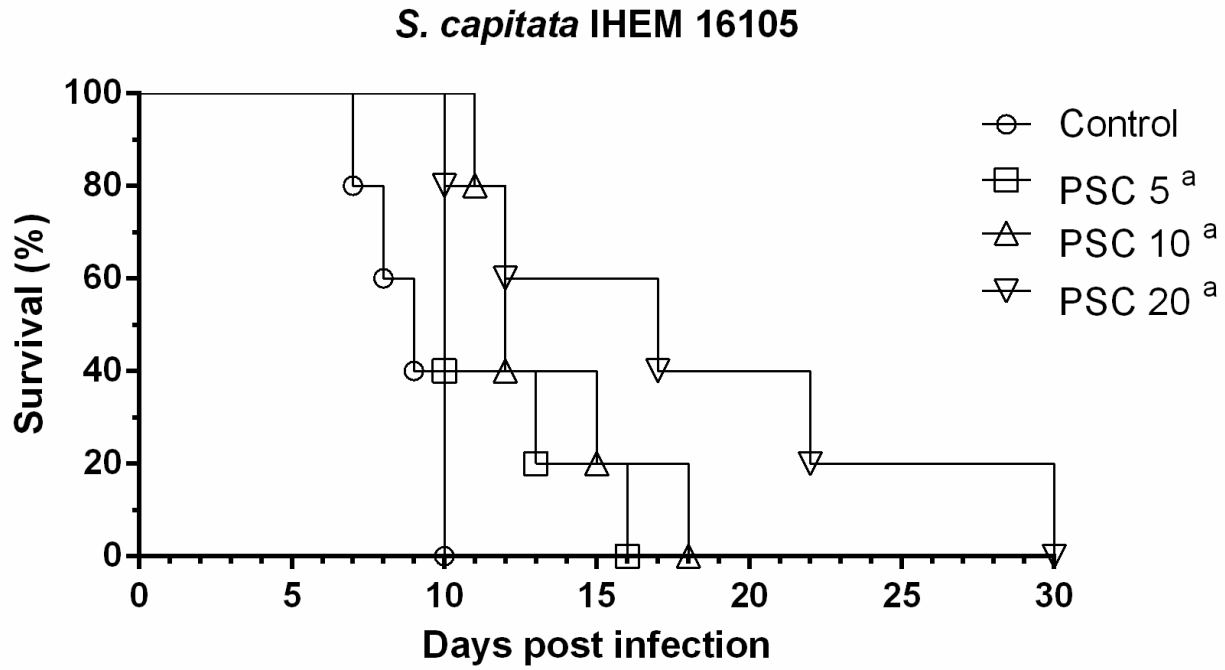
Figure 4. (1→3)-β-D-glucan serum levels in mice infected with *S. capitata* A) strains IHEM 5666 and IHEM 16105; group control, PSC 5, 10 or 20 mg/kg BID. B) strains IHEM 5665, IHEM 5091, IHEM 6803 and IHEM 16109; group control, PSC 10 mg/kg BID. Horizontal line indicates the cut- off positive (80 pg/ml). [a] $P \leq 0.028$ versus control; [b] $P \leq 0.028$ versus PSC 5; [c] $P \leq 0.028$ versus PSC 10.

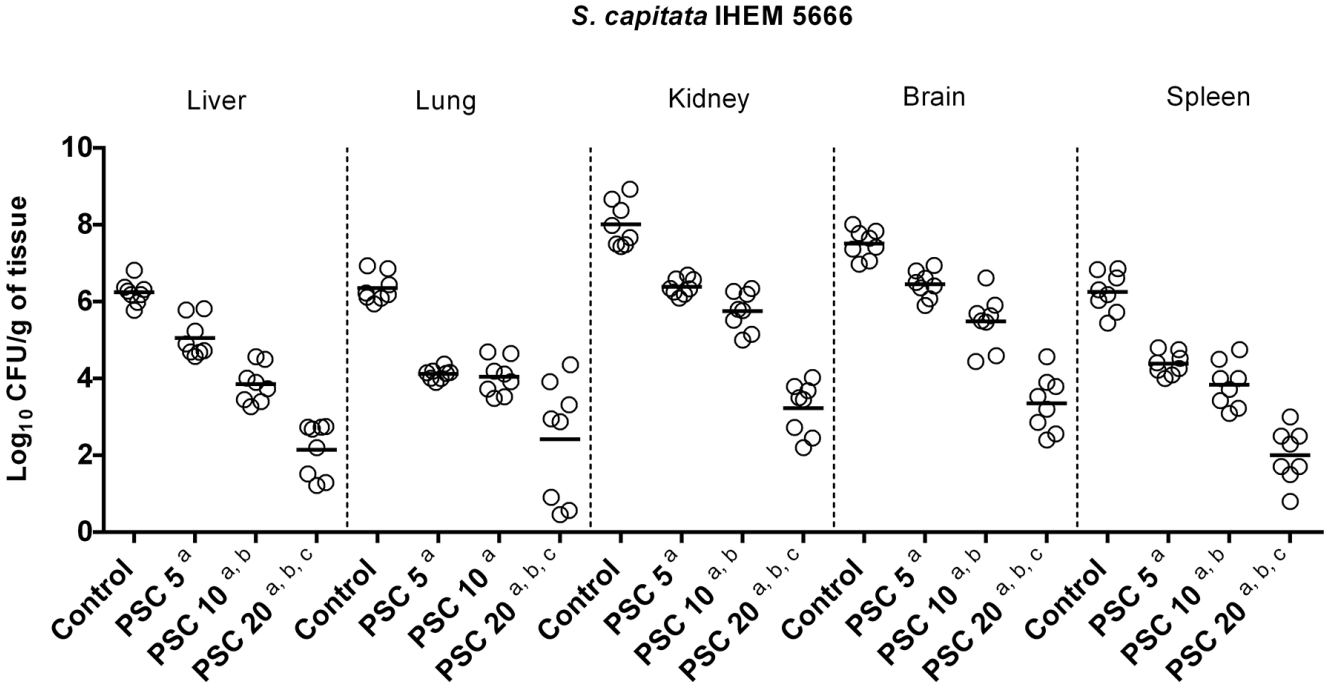
Figure 5. Histological findings in kidneys of immunosuppressed mice infected with *S. capitata,* 7 days post-infection (strain IHEM 16105). A-B corresponds to control mice showing massive invasion of renal parenchyma by hyphae without inflammatory response

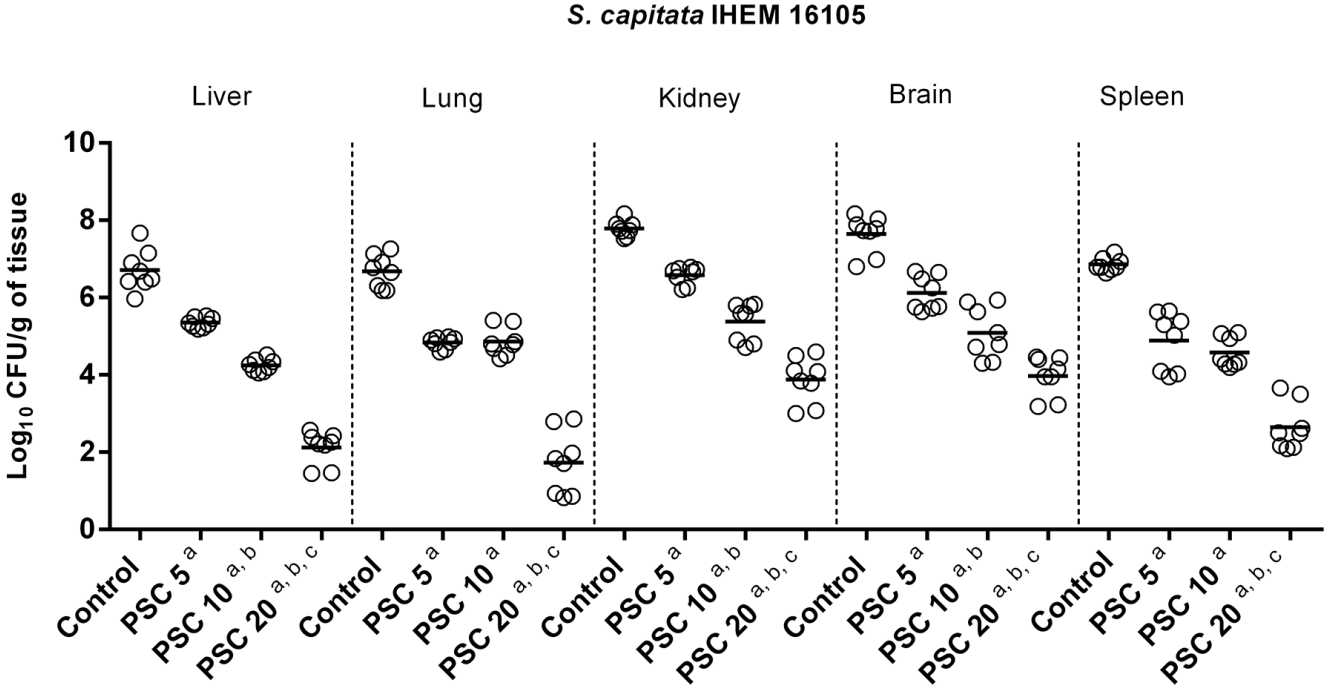
or necrosis. C-D mice treated with PSC 10 showing decrease of hyphae in renal parenchyma level and E-F mice treated with PSC 20 showing less presence of hyphae within renal tubules. A-C-E stain PAS x 400. B-D-F stain GMS x 400.

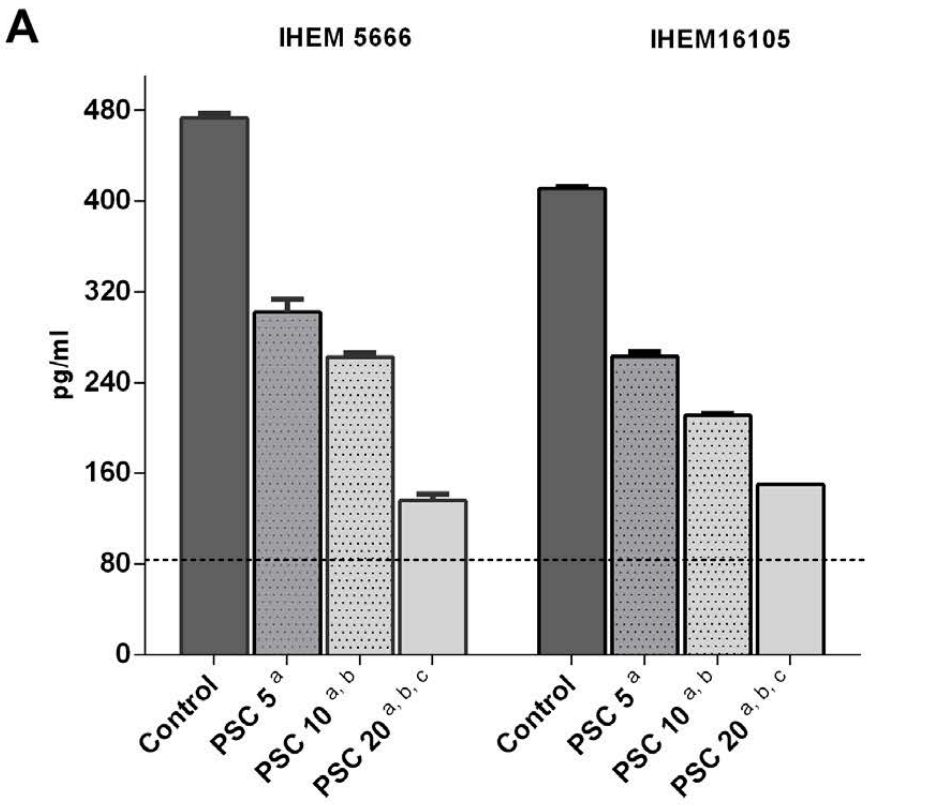


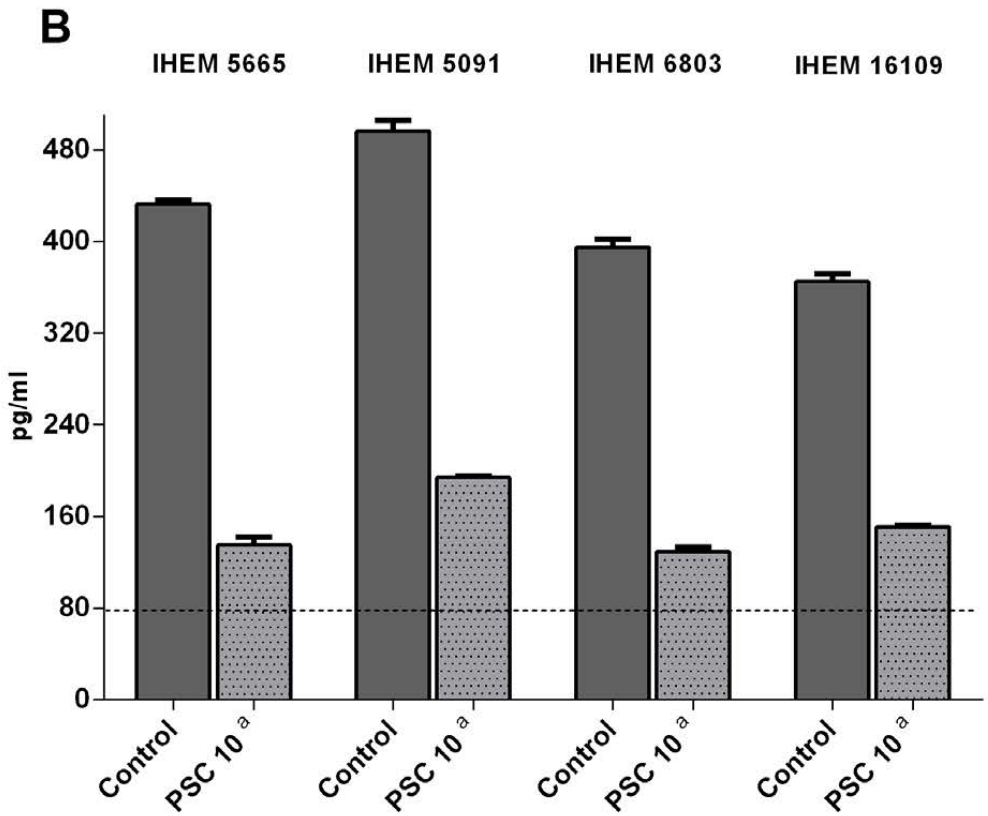


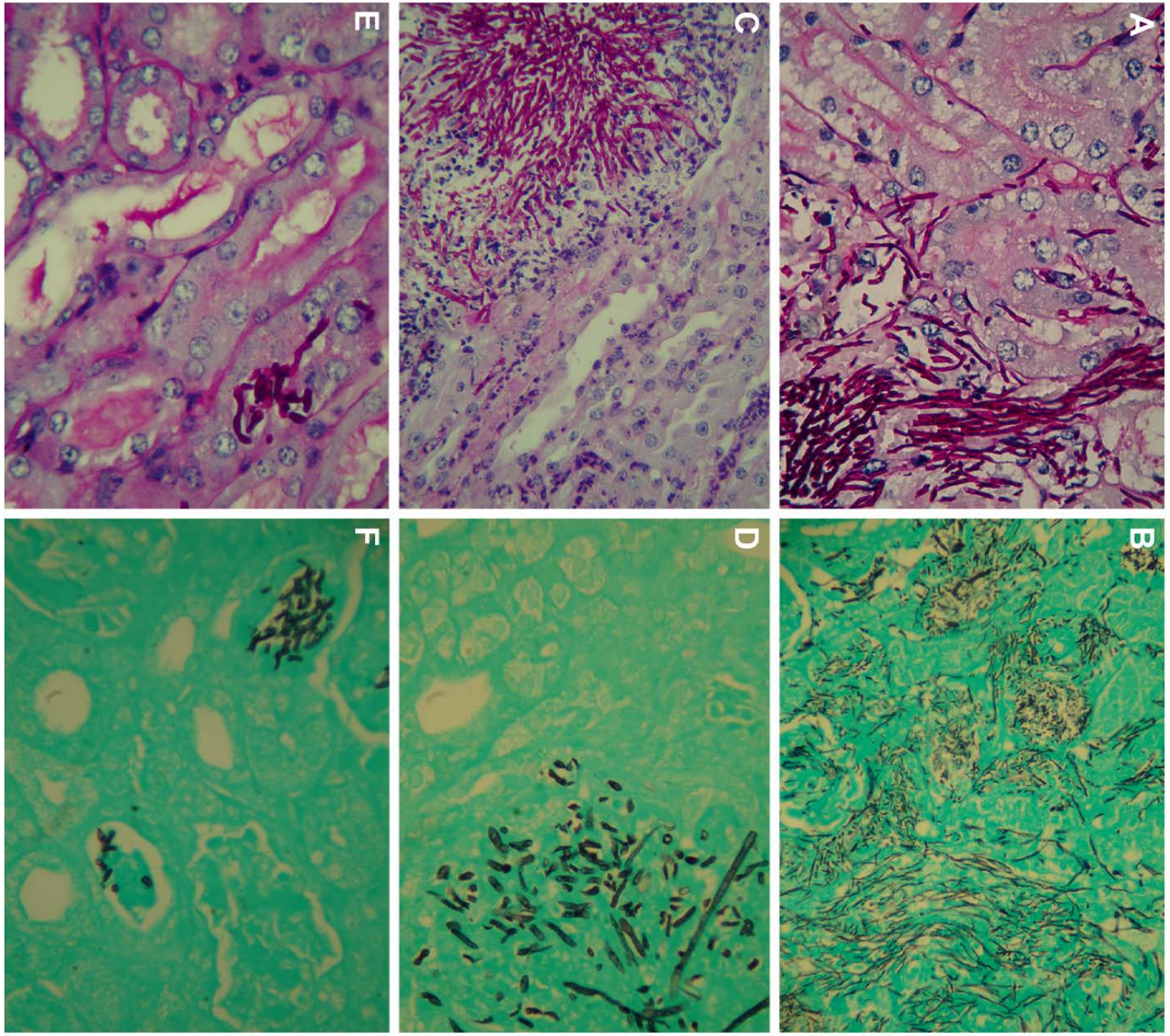








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