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The serum glucan level and pathological changes of antifungal treatment for lower respiratory tract infection of *Candida albicans*

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Abstract

Due to the fact that *Candida albicans* colonizes in the upper respiratory tracts of healthy people, whether or not its isolation from airway secretions is sufficient to warrant treatment remains controversial. The animal models of immunosuppressive rats with pulmonary candidiasis were established by the intratracheal inoculating suspensions of *C. albicans,* and the animals were divided into the following three groups: (1) antifungal treatment group, (2) saline control group, and (3) blank control group. We noted the following in our studies: (1) The fungal load of the saline control group gradually increased such that it was higher than those of the antifungal treated group and was significant from the fourth day of treatment ($P < 0.01$). (2) The serum (1,3)-β-D-glucan (BG) in the saline control group also gradually increased so that it was significantly higher than found with the treated group by the sixth day of treatment ($P < 0.05$), and in fact, the rank of pulmonary colony count and BG in the two groups at different time points showed an almost perfect linear correlation. (3) The median survival period of the rats in the antifungal treated group and saline control group was 15 and 8 days respectively, no rats died in the blank control group. (4) The lung lesions from the saline control group gradually became more aggravated than those in the antifungal treated group; no significant pathological changes were found in the blank control group. Antifungal treatment (*micafungin*) is capable of efficaciously decreasing the lung fungal burden, and continuous monitoring of BG is useful for the evaluation of therapeutic effect of antifungals. Infection of *C. albicans* with associated pathological damage implies the need for antifungal therapy.

Key words: *Candida albicans*, infection, therapy, animal experiment, (1,3)-β-D-glucan, pathology.

Introduction

With the recent increase in the application of broad-spectrum antibiotics, immunosuppressants, anticancer drugs, the progress of a variety of interventional therapy and life support methods, the epidemic of AIDS in some areas, as well as wide organ and bone marrow transplantation, fungal infections become more severe and may even lead to lethal outcomes in immunocompromised patients [1]. *Candida albicans* ranks fourth in the etiology of nosocomial infections, and its reported association with such infections increases every year [2]. Pulmonary candidiasis is a type of lung and bronchial tract infection generally caused by *Candida albicans*. Respiratory secretions, such as sputum smear and culture, are the main methods for detecting pathogenic microorganisms in respiratory infections. However, due to the fact that the airway is connected with the outside world, which favors pollution and opportunistic pathogens, it is still controversial whether or not the *C. albicans* detected in airway secretions require treatment. Some researchers have proposed that, due to the fact that *C. albicans* colonizes in the upper respiratory tract of healthy people, and there is also the possibility of contamination, treating increases the economic burden on patients and possibly induce the generation of drug-resistant strains. In 2009, the Infectious Diseases Society of America updated the Clinical Practice Guidelines for the Management of Canadidiasis and stated that *Candida* isolated from the respiratory secretions does not always indicate invasive candidiasis nor does it indicate the need for antifungal therapy [3]. In addition, a number of prospective and retrospective studies (including autopsies) have shown that there is little clinical significance in the recovery of *Candida* from respiratory secretions (including bronchoalveolar lavage fluid) used to establish diagnosis of pulmonary candidiasis [4–6]. On the other hand, some researchers believe that, due to the tense situation of the doctor-patient relationship at present, the positive detection of *Candida* in respiratory secretions without a treatment could contribute to legal actions taken by the patient against the clinician. Other questions remain as well, such as does the isolation of *C. albicans* from the lower respiratory tract require treatment? Does the treatment help? It is clear that there is a lack of research in this area. Different from the previous sepsis rat model involving vein injection of *C. albicans*, in the present study an alternative method involving inoculation of *C. albicans* through the respiratory tract was adopted to simulate the infection process in humans; then the burden, pathology, and prognosis of infected rats to *C. albicans* were determined.

Materials and methods

Preparation of *Candida albicans*

The *Candida albicans* strain ATCC90028, which is sensitive to micafungin, with a minimum inhibitory concentration (MIC) of 0.031 mg/l [7] was purchased from the Guangdong Culture Collection Center.

The strains

The *Candida albicans* strains were inoculated onto a Sabouraud Dextrose agar plate grown at 37°C for 48 h, and then the surface rinsed with 10 ml of saline to collect the spores, with the latter filtered through eight layers of sterile gauze to remove the hyphae. The spore suspension was rinsed twice with saline and counted using a microscope equipped with a hemocytometer. The low concentration of spores (5×10^8 cfu/l) was used to measure the treatment effect, while a high concentration of fungi (5×10^{12} cfu/l) was employed for survival analysis. The pre-experiment confirmed that the LD50 of *C. albicans* when treated with methylprednisolone combined cyclophosphamide amide immunosuppression was approximately 5×10^{11} cfu/l. The viable cells were subjected to 10-fold serial dilutions to quantitatively detect the activity of the spores.

Animals

Eight-week-old male Sprague Dawley (SD) rats with an average weight of 200 ± 20 g were purchased from the Experimental Animal Center of Sun Yat-sen University, (SCXK (Guangdong) 2013–0081) for use in the study. The rats underwent adaptive feeding for three days with the standard feed and free access to drinking water before the experiment. In the therapeutic effect experiments, a total of 66 male SD rats were randomly divided into three groups: (1) the antifungal treatment group ($n = 30$), with immunosuppressive therapy, intratracheal inoculation of *C. albicans*, and micafungin; (2) the saline control group ($n = 30$), with immunosuppressive therapy, intratracheal inoculation of *C. albicans*, and saline; and (3) the blank control group ($n = 6$), with immunosuppressive + intratracheal inoculation of saline. In the survival analysis experiments, 36 SD rats were randomly divided into three groups of 12, and the grouping was the same as the therapeutic effect experiments.

Establishment of immunosuppressive model

The immunosuppressive rat model was established according to a previous report [8]. Specifically, 20 mg/Kg/d of methylprednisolone (Pfizer) was constantly subcutaneous

injected two weeks prior to the beginning of inoculation. Then 50 mg/Kg/d of cyclophosphamide (Shanxi Pude Pharmaceutical Co., Ltd.) was also injected to strengthen the immunosuppressive effect at −4 and −1 days prior to inoculation. In addition, 10 mg/Kg/d of Cravit (Daiichi Sankyo Pharmaceutical Co., Ltd.) was intraperitoneally injected to prevent bacterial infections. Fifteen rats were randomly selected before and after immunosuppression, and then their tail vein blood, 0.5 ml from each rat, was collected in EDTA anticoagulated tubes and subjected to blood routine examination.

Two days after immunosuppression, the rats were killed with intraperitoneal injection of 10% chloral hydrate (3.0 ml/kg) and fixed on the fixation plate. After their neck hair was fleeced, the rats were disinfected with 75% alcohol. The tracheotomy was performed at aseptic condition to expose the upper trachea of the rats. A 1 ml syringe was punctured through the trachea, to perform injection of the *C. albicans* solution into the trachea. After injection, the rats were kept in the vertical position for 3–5 minutes, followed by suture of the skin incision. For the blank control group, equal amounts of saline were injected in place of the *C. albicans* solution.

Administration of antifungal drug

The rats in the antifungal treatment group were intravenously injected with 5 mg/Kg/d of micafungin (Astellas Pharma Ltd.) in a 0.2 ml saline solution for 7 days and once per day. The rats in the saline control group were intravenously injected with equal amounts of saline.

Determination of therapeutic effect

In the therapeutic effect experiments, all the rats in the blank control group were killed 2 days after intratracheal inoculation of saline, while the rats in the untreated and antifungal treatment groups were killed before intratracheal inoculation, as well as at 2, 4, 6, and 7 days after intratracheal inoculation, with six rats for each time point. Their blood was collected and analyzed using the MB-80 microbial dynamic rapid detection system (Jinshanchuan Science and Technology Development Co. Ltd., Beijing), and the EKT-5M Set fungal dynamic detection kit for detecting the serum (1,3)-β-D glucan (BG) level, in accordance with the instructions. The right lung was removed aseptically, homogenated with 5 ml of saline, and diluted in 10-fold gradient series. Two hundred ul of solution was evenly inoculated on the Paul's agar and cultured at 37°C for 48 hours, followed by counting of the colony (cfu/g lung). The left lung was fixed in a 10% formalin solution, and the paraffin sections were subjected to H&E staining for histopathological examination.

Survival analysis

After treatment, the animals in each group were observed for an additional two weeks, and the deaths of the animals were recorded for survival analysis and calculation of the median survival.

Statistical analysis

The SPSS 13.0 statistical software was used for all analyses. The results of the blood testing and (1,3)-β-D glucan antigen content were shown as mean ± SD ($\bar{x} \pm s$), and the fungal colony number was lgcfu ± s. The Kaplan-Meier method was used for the survival analysis. The normally distributed measurement data between the two groups were compared using *t*-tests, and the correlation analysis used the Pearson correlation analysis. The nonnormal distributions or variances of missing data between the two groups were compared using Wilcoxon rank sum tests, and for the correlation analysis Spearman rank correlation was adopted. The level of $P < 0.05$ was considered statistically significant.

Results

Therapeutic effect: colony counts

After receiving *methylprednisolone* and *cyclophosphamide* immunosuppression, the rats were characterized by listlessness, loss of appetite, and inactivity, which were highly promoted after intratracheal inoculation of *Candida* bacteria, with the additional feature of shortness of breath. The antifungal *micafungin* treatment ameliorated these symptoms, by means of increased appetite and activity. After subcutaneous injection of *methylprednisolone* and intraperitoneal injection of *cyclophosphamide*, the white blood cell (WBC), the neutrophil, and lymphocyte counts decreased significantly, thus indicating that the immunosuppression procedure was successful.

After intratracheal inoculation of *C. albicans*, the lung tissue from the rats in the antifungal treatment and saline control groups were collected and planted on the Paul's agar medium and cultured at 37°C for 48 hours, resulting in multiple white cheese-like colonies, whereas the lung homogenates cultures of the blank control group were negative. All of these results indicate the successful establishment of *C. albicans* pneumonia in immunosuppressed rats.

In addition, the fungal load of the saline control group increased gradually with the prolongation of infection, while the fungal load of the antifungal treated group decreased. Their respective results showed significant differences beginning from the fourth day of the treatment ($P < 0.05$, Table 1).

Table 1. Comparison of the pulmonary fungal burden between the antifungal treated group and the saline control group (lgcfu/g±s).

Date	Antifungal treated group	Saline control group	*P* value
Before treated	7.23 ± 0.21	7.20 ± 0.29	$P = 0.81$
2 day post inoculation	6.77 ± 0.22	7.08 ± 0.35	$P = 0.09$
4 day post inoculation	5.28 ± 0.51	7.44 ± 0.62	$P < 0.01$
6 day post inoculation	4.14 ± 0.41	7.76 ± 0.47	$P < 0.01$
7 day post inoculation	3.33 ± 0.40	7.88 ± 0.32	$P < 0.01$

Therapeutic effect: level of serum BG

The serum BG of the blank control group was below the detection limit and was negative. The serum BG of the saline control group increased gradually with the prolongation of infection, whereas the serum BG of the treated group decreased. Their respective results showed significant differences beginning from the sixth day of treatment ($P < 0.05$, Table 2).

Therapeutic effect: correlation between pulmonary colony count and serum BG

The ranks of the pulmonary colony count and serum BG of the treated and saline control groups at different time points showed an almost perfect linear correlation ($P < 0.01$). The rank correlation coefficient was 0.521, which indicated that the serum BG may be an index for the therapeutic effect of *micafungin*.

Survival of rats in each group

None of the rats in the blank control group died. The median survival period of the rats in the antifungal treated group was 15 days, and in the saline control group it was 8 days. After Log-rank testing, the results of the two groups showed significant differences ($P < 0.01$). In addition, the survival curves of the antifungal treatment and saline control groups were different ($P < 0.01$), which indicated that the *micafungin* treatment helped to extend the lifetime of the immunosuppressive *C. albicans* pneumonia rats (Fig. 1).

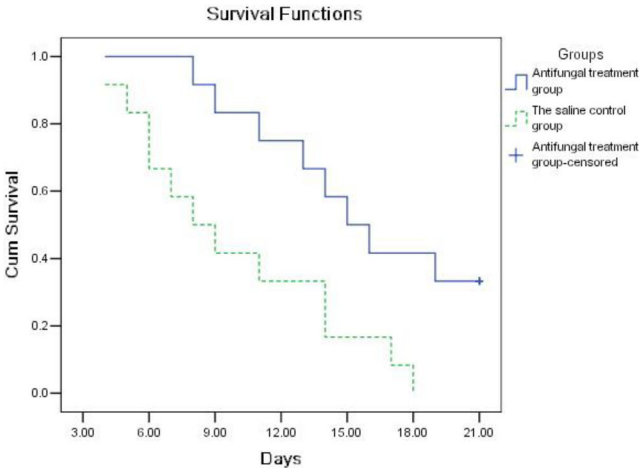

Figure 1. The survival curves between the antifungal treated group and the saline control group.

Pathological changes of lung

Between the intratracheal inoculating suspension of *C. albicans* and the *micafungin*/saline treatment, the HE staining of the lung tissue showed scattered bleeding, a small amount of inflammatory cells, interstitial edema, and widening. The lung tissue lesions in the saline control group gradually became more and more aggravated, with significantly widened alveolar septa and obscured alveolar structure at 7 days after infection. On the other hand, the antifungal treated group showed less severe pathological changes, and these changes were not found in the blank control group (Fig. 2).

Discussion

Candida albicans is a conditional pathogenic fungus, which generally exists in the body as a yeast phase. When the

Table 2. Comparison of level of serum *(1,3)-β-D-glucan* (BG) between the antifungal treated group and the saline control group ($\bar{x} \pm s$, pg/ml)

Date	Antifungal treated group	Saline control group	*P* value
Before treated	225.91 ± 239.92	255.89 ± 209.32	$P = 0.937$
2 day post inoculation	229.04 ± 246.52	217.60 ± 240.63	$P = 0.818$
4 day post inoculation	208.97 ± 179.31	300.45 ± 319.89	$P = 0.394$
6 day post inoculation	91.18 ± 102.32	407.44 ± 252.62	$P < 0.05$
7 day post inoculation	80.49 ± 64.72	465.22 ± 229.23	$P < 0.05$

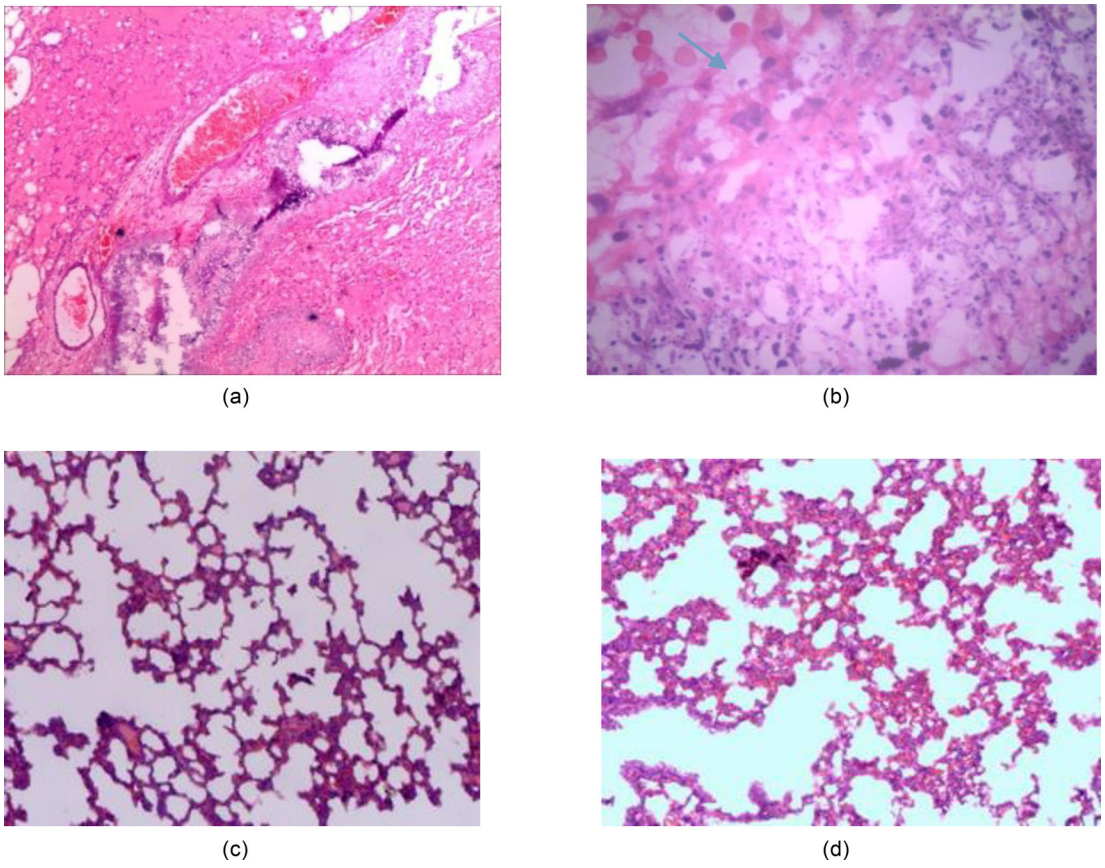

Figure 2. Pathological changes of lung tissue among differnt group.

body's immune function is suppressed or suffers from microflora imbalance, *C. albicans* converts from a yeast to mycelial phase, and in so doing, may initiate infections. The morphology of *C. albicans* affects its pathogenicity, and its mycelium phase becomes more aggressive and exhibits higher tissue penetration, whereas the yeast phase causes hematogenous infections [9]. A difficulty in diagnosis of *Candida* infection is distinguishing between routine *Candida* colonization and the yeast as the cause of infection, which is of great significance for clinicians when performing antifungal therapy. At present, the diagnosis of *Candida* pneumonia requires not only the appropriate clinical manifestations but also pathological evidence for *Candida* invasion in lung parenchyma [3]. Clinically, due to the unstable conditions of patients, as well as pathological bleeding, pneumothorax and other risks, invasive methods are used less and less frequently in the diagnosis of candidiasis. Therefore, accurate diagnoses of *Candida* pneumonia are very rare, as is the therapeutic effect of antifungal therapy on pulmonary candidiasis. In the present study, a rat model of immune suppression and pulmonary infection of *Candida albicans* was established to study the prognosis of anti-fungal treatment on candidiasis. Between the intratracheal inoculating suspension of *C. albicans* and *micafungin*/saline treatment, the rats in the antifungal treatment group and saline control group both showed positive detections of *C. albicans*, with pathologic evidences for *C. albicans* transiting from yeast phase to mycelium phase, as well as invasion of lung tissue, which indicated that the established animal model reached a diagnosis of pulmonary candidiasis histological criteria, but was not just simply infected. In addition, the colony counts for cultured lung homogenates were used as an indicator to determine the drug efficacy [10], together with the increased serum BG level, and all of these results confirmed the presence of deep fungal infection.

Micafungin immunotherapy was able to effectively reduce kidney fungal burden in an immunosuppressive systemic *Candida glabrata* infection mouse model [11]. However, in an invasive pulmonary aspergillosis mouse model, it was unable to reduce pulmonary fungal burden [12]. The results of the present study showed that the lung colonies of the rats in the anti-fungal treatment group decreased after treatment, whereas those in the saline control group tended to increase, with significant differences at the 4th day ($P < 0.05$), indicating that antifungal therapy reduces pulmonary fungal burden in rats. These results were consistent with the other authors [11], but not consistent with

those [12], which may be due to the inhibitory effect of *micafungin* on *Aspergillus* and the bactericidal activity of *micafungin* on *Candida* [13].

BG is an important component of many fungal cell walls. When invading blood or deep tissue, the fungus will be engulfed by neutrophils and digested phagocytic cells, and release BG from the cell walls out into the blood or other tissue fluid. Studies have shown that, when different methods and boundary values were properly used, the diagnosis of candidemia has a high sensitivity (64%–90%) and specificity (73%–100%), with a negative predictive value as high as 73%–97% [14,15]. A hematologic malignancies study showed that the diagnosis of candidemia by BG detection significantly reduced time cost in comparison to clinical, radiological, and pathological criteria [16], thus making early diagnosis of invasive candidiasis possible. The present study found that both groups of rats, after lung infection of *C. albicans*, had rapidly increased BG, which will be useful for early diagnosis; however, some researchers hold different views. The serum BG levels pre-treatment and at the early stage of treatment had little significance for the clinical prognostic [17]. Moreover, the early negative serum BG could not be used as a sign of effective anti-fungal treatment, as it usually takes several weeks to reduce to normal level [18,19]. The present study found that the serum BG levels in the saline control group were gradually increased but decreased after micafungin treatment, and the difference between the two groups became statistically significant at 6 days after treatment ($P < 0.05$). In addition, the colony counts and serum BG level had a linear relationship, indicating that the continuous monitoring of serum BG level can be an initial assessment of the effect of antifungal therapy. This is consistent with the findings of a nonneutropenic *Candida* infection model proposed [20].

BG is present in the cell walls of most pathogenic fungi but not in zygomycetes or *Cryptococcus* [21]. Under the conditions of infections caused by these two fungi, it is no use for diagnosis and evaluation of the therapeutic effects by monitoring the serum BG level. Furthermore, it has limitations for monitoring serum BG level to predict the effect of antifungal therapy, and it must combine with clinical symptoms, signs, and other laboratory tests to perform a comprehensive judgment.

The results of this study showed that the median survival of the antifungal treatment group and saline control were 15 days and 8 days, respectively, which is a significant difference ($P < 0.05$). This indicates that antifungal therapy can effectively extend the survival of the rats in the immunosuppressive pulmonary *C. albicans* infection model and improve prognosis, which is consistent with the results of previous studies [11]. The conclusions showed that antifungal treatment is capable of efficaciously decreasing the lung fungal burden of immunocompromised rats with pulmonary *Candidiasis albicans*. The continuous monitoring of serum (1,3)-β-D-glucan is useful for the evaluation of therapeutic effect of antifungal medicine. Antifungal therapy (micafungin) can alleviate the inflammation of the lung tissue of immunocompromised rats with pulmonary candidiasis, prolong their survival, and improve their prognosis. The infection of *C. albicans* in the lower respiratory tract and presence of pathological damage implies the need for antifungal therapy.

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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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