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Effects of fluconazole on the clinical outcome and immune response in fungal co-infected tuberculosis patients

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ABSTRACT

With overuse of the broad-spectrum antibiotics, the pulmonary fungal infection increasingly becomes the most common complication associated with senile pulmonary tuberculosis (TB) and attracts intensive attentions from clinicians. Here we presented the retrospective analysis of impact of fluconazole treatment on the clinical outcome and immune response in fungal co-infected tuberculosis patients. A randomized, double-blind, placebo-controlled trial of fluconazole (100 mg per day for consecutive weeks) in fungal-positive senile tuberculosis patients was conducted in our hospital. Peripheral eosinophil counts were computed by the automatic hematology analyzer. The secretory inflammatory cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α and chemokines chemokine C-X-C motif ligand (CXCL)9, CXCL10, CXCL11 were determined with enzyme-linked immunosorbent assay kits. The peripheral T helper 1 cells (Th1) and regulatory T cells (Treg) population were analyzed by flow cytometry. None of significant difference in respect to baseline TB score was observed between placebo and fluconazole groups. Administration of fluconazole significantly stimulated eosinophils population and secretion of inflammatory cytokines IFN-γ and TNF-α. Simultaneously, the peripheral Th1% and chemokines including CXCL9, CSCL10, CXCL11 were markedly induced in response to fluconazole treatment. Fungal infection significantly affected host immunity during tuberculosis which was effectively reversed by fluconazole treatment.

1. Introduction

Tuberculosis (TB) is the major public health issue with significant morbidity and mortality worldwide [1]. In recent years, the prevalence of TB and the prognosis of the disease have been greatly improved [2]. However, with the effective medical interventions and the overall aging trend in population age structure, morbidity of TB has been tremendously transited to the elder population [3]. Simultaneously, the senile TB patients are more susceptible to a variety of pathogenic factors due to poor nutritional status, declined immune function, accumulative lung tissue impairment [4]. Clinically, with the overuse of broad-spectrum of antibiotics, pulmonary fungal infection has become one of the most common complications in elderly TB patients, and increasingly attracts more and more attention from the specialist [5]. In order to summarize the prevalence, risk factors, clinical features, diagnosis and treatment of pulmonary tuberculosis patients with fungal infection, here we perform the comprehensive retrospective investigation in respect to fluconazole therapy, the first-generation triazole antifungal medication used for array of fungal infections including candidiasis, blastomycosis, coccidiodomycosis, cryptococcosis, histoplasmosis, dermatophytosis and pityriasis versicolor [6]. In total, 261 cases of elderly patients with pulmonary tuberculosis and pulmonary fungi infection were enrolled in the placebo-controlled clinical study, and the primary and secondary outcomes were cautiously evaluated.

The protective immunity in TB patients has been proposed that predominantly relies on T-helper 1 (Th1) $CD4^+$ T cells which produces the inflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, along with granule-associated cytolytic effector molecules producing cytolytic T cells (CTLs) [7]. The secretory IFN-γ subsequently activates the antimycobacterial action in macrophages to effectively kill or restrict the TB pathogens [8]. Fungal co-infection has been speculated that complicates this disease and modulates the protective Th1-response against TB to some extent through the influence on the Th1/Th2 balance with enhanced Th2 dominance and activation of regulatory T cells (Tregs), which in turn stimulated the production of inhibitory cytokines against Th1 response during the pathogenesis of

Abbreviations: Tuberculosis, (TB); T-helper 1, (Th1); body mass index, (BMI); mid-upper arm circumference, (MUAC)

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TB [9]. The complicated interactions between active TB and fungal infections and consequent influences on therapeutic outcomes are still elusive. Therefore, we sought to comprehensively interrogate the immune response in fungal co-infected senile TB cases in response to fluconazole via characterization of peripheral Th1 and Treg sub-population and quantification of serous contents of inflammatory cytokines and chemokines. Our data for the first time demonstrated that fluconazole treatment improved clinical outcome in respect to TB status. However, the immune response was tremendously stimulated as indicated by the increase of Th1 population in plasma and corresponding inflammatory cytokines. In addition, the serous chemokines including chemokine C-X-C motif ligand (CXCL)9, CXCL10 and CXCL11 were greatly up-regulated, which might underlie the Th1 cells enrichment in response to fluconazole treatment. We further proposed that fluconazole-stimulated TNF-α might contribute to the regulation of array of chemokines.

2. Methods

2.1. Study participants

Totally, 261 consecutive patients diagnosed with pulmonary TB from June 2013 to June 2017 at Cangzhou Central Hospital were enrolled in this study with written informed consent. The diagnostic criteria were based on either acid-fast bacilli (smear-positive TB) or chest X-ray (CXR) results according to the World Health Organization guidelines. The fungal co-infected senile patients were randomly allocated to fluconazole treatment (100 mg/day for four consecutive weeks) or identical placebo tablets to make the contents blind to the patients. Investigators who conducted experiments were blind to patient group assignment. The protocol was approved by the Ethics Committee of Cangzhou Central Hospital.

2.2. TB score

The TB score (0–13 points) was computed based on the clinical information of TB patients collected from the structured questionnaire and composed of signs and symptoms each contributing one point (cough, haemoptysis, chest pain, dyspnoea, night sweating, anaemic conjunctivae, lung auscultation finding, tachycardia ($\geq$100/min), temperature ($\geq$37 °C), body mass index (BMI) $\leq$18 kg/m^2, BMI $\leq$16 kg/m^2, mid-upper arm circumference (MUAC) $\leq$220 mm, and MUAC $\leq$200 mm).

2.3. CXR evaluation

CXR grading of pulmonary TB was performed in accordance with the guideline from the National Tuberculosis Association of the USA as normal, minimal, moderate and far advanced TB (American Thoracic Society, 1961), which was translated to a semi-quantitative scale for statistical analysis. The CXRs were evaluated by three experienced radiologists independently.

2.4. Determination of eosinophil counts

The venous blood was collected from TB patients receiving fluconazole administration for 4 consecutive weeks. The absolute counts of eosinophil in peripheral blood was determined in cells/mm^3 from the value of total and differential white blood cell counts obtained from the automatic hematology analyzer (Cell Dyn 1800, Abbot, USA).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The peripheral blood from TB patients treated with either fluconazole or placebo was collected. The hemocytes were completely removed by centrifugation and serum was collected and cryopreserved at for −80 °C ELISA analysis. The serous levels of IFN-γ, TNF-α, CXCL9, CXCL10 and CXCL11 were measured with the commercially available ELISA kits (IFN-γ, ab46025; TNF-α, ab46087; CXCL9, ab100595; CXCL10, ab83700; CXCL11, ab187392 from Abcam, MA, USA) following the manufacturer's instructions.

2.6. Flow cytometry

The peripheral blood mononuclear cells (PBMCs) were isolated using the Dynabeads Untouched Human T Cells Kit (ThermoFisher, UT, USA) in accordance with the manufacturer's instruction and cryopreserved at −80 °C in FCS containing 10% DMSO. The cell viability was evaluated with trypan-blue exclusion assay prior to further analysis. PBMCs were stained with fluorochrome-labeled monoclonal antibodies CD4-Alexa Fluor 488 (MHCD0420, ThermoFisher, UT, USA) and CD25$^-$ APC650 (17-0259-42, ThermoFisher, UT, USA), followed by fixation and permeabilization using cytofix-cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA) and intracellular staining with IFN-γ- PE-eFluor 610 (61-7311-82, ThermoFisher, UT, USA) and Foxp3-eFluor 570 (41-4777-82, ThermoFisher, UT, USA). Th1 cells were defined as the CD4$^+$IFN-r$^+$ population and Treg were defined as CD4$^+$CD25$^+$Foxp3$^+$ T cells. Flow cytometry data were acquired on the FACSCalibur (BD Biosciences, USA) with Cell-Quest acquisition software and analyzed by Flowjo 7.6.5.

2.7. Statistical analysis

Data was acquired from at least three independent experiments. Data analysis was performed with SPSS 23.0 software and expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) followed by Turkey's test was employed for statistical comparison. The statistical significances were calculated as P values, and $P < 0.05$ was considered statistically different.

3. Results

3.1. Study population

A total of 261 newly diagnosed pulmonary TB patients with fungal co-infection were enrolled in this investigation. The fungal-positive TB patients were randomly allocated to fluconazole (135) or placebo (126) treatments (Table 1). None of obvious adverse effects were observed due to fluconazole treatment during our study.

3.2. Baseline characteristics

In our enrolled subjects, none of significant baseline differences between the fluconazole and the placebo groups in respect to the severity of average TB score (8.1 ± 2.6 points in fluconazole group versus 7.8 ± 2.8 points in placebo group). Likewise, the percentage of

Table 1
Baseline characteristics of tuberculosis patients.

	Placebo (N = 126)	Fluconazole (N = 135)	*P* VAUE
Ages (years)	66.8 ± 14.8	67.6 ± 15.2	NS
Sex			
Males	72	81	NS
Females	54	54	NS
TB score (points)	7.8 ± 2.8	8.1 ± 2.6	NS
Sputum SP (%)	64	68	NS
CXR			
Normal (%)	5	6	NS
Mild (%)	20	21	NS
Moderate (%)	45	30	NS
Far advanced (%)	30	43	NS

SP, sputum positive; CXR, chest X-ray.