ORIGINAL CONTRIBUTION

The Effect of Fluconazole Treatment on Tumor Necrosis Factor-α Production in Murine Candidiasis

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That systemic candidiasis increases TNF-α has been proved in various studies. In our study, we investigated if fluconazole too, which is used in treatment, has an additional effect on the increase of TNF-α. On the sixth day of the experimental infection, it was measured that TNF-α levels of candidiasis was 699 ± 60 pg/ml whereas it was 683 ± 35 pg/ml in the mice that were treated with fluconazole. TNF-α levels were measured by enzyme-linked immunosorbent assay. Due to the fact that no statistically significant difference was found between them (p > .05) it was thought that fluconazole did not have an additional effect on the increase of TNF-α in candidiasis.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) increases as an essential cytokine during sepsis that is caused by Candida infections. Candida species activate both the humoral and the cellular immune systems. Being the main protector of Candida infections, cellular immunity consists of macrophages, neutrophils, mononuclear phagocyte system and natural killer cells, all orchestrated by T cells [1]. After activated macrophages are stimulated by lipopolysaccharide, they become the largest source of TNF-α. Other than activated macrophages, mast cells, polymorphonuclear leukocytes, keratinocytes, astrocytes, microglia cells, smooth muscle cells, and tumor cells also release TNF-α [2]. It has been demonstrated that similar to the increase of TNF-α levels during Candida infections, some antifungal agents also increase TNF-α level as a side effect. Especially during the treatment of amphotericin B (Amp B), systemic side effects are frequently observed. The increase of TNF-α level, which can be both protective and harmful to the organism, can be seen among the side effects. Fluconazole is the most frequently used azole-derived agent, and it is an antifungal with minimum side-effects [3]. Whether fluconazole activates TNF-α or not has been investigated in a small number of in vitro [4, 5] and in vivo studies [6, 7].

The purpose of our study is to measure TNF-α level on the experimental model of murine candidiasis and to investigate if fluconazole has an effect on TNF-α level or not.

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Abbreviations: Amp B, amphotericin B; OD, optic density; PBS, phosphate buffered saline; TNF-α, tumor necrosis factor-α.
MATERIALS AND METHODS

Experimental animals

Female, six- to eight-week-old, 20 to 25 g BALB/c mice, maintained from Hifzisihha Serum Farm, Ankara, Turkey where the mice were bred under standard conditions.

Organisms

*C. albicans* ATCC 26555 (Kukens, Istanbul, Turkey) reference strain were used.

The preparation of yeast suspensions

Four or five colonies of *Candida albicans*, which had been formed in sabouraud dextrose agar (SDA, Oxoid) after 48-hr incubation, were suspended in 5 ml of sterile, pyrogen-free, 0.9 percent NaCl. Organisms were harvested by low-speed centrifugation (1500 xg), washed three times in sterile saline, counted with a hemocytometer and suspended to $3 \times 10^8$ cell/ml concentration. Mice were challenged by tail vein injection with 0.2 ml of suspension 99 percent of which was blastoconidia [8, 9].

Measurement of Serum TNF-α

Blood was collected from the heart. Serum TNF-α levels were quantified by using enzyme-linked immunosorbant assay kit called Biosource CytoscreenTM Mouse TNF-α New Format (United States) [10].

Demonstration of Candida infection

Both of the kidneys were isolated and incubated to show *Candida* infection. Both of the kidneys homogenized in 10 ml phosphate-buffered saline were inoculated in equal amounts into SDA plates which contain 100 IU penicillin and 10 μg streptomycine. The same homogenized suspensions were also inoculated into blood agar plates in order to eliminate the presence of bacterial infections. All of the yeast colonies were counted and documented after a 48-hr incubation period [8].

Statistical analysis

All statistical analysis were calculated by the help of the computer program, SPSS 9:01. The graph (Figure 1) which shows the optic density (ODs) values that correspond to the standards of TNF-α, was drawn with regression analysis method ($y = -35.189 + 468.621 \times$ OD and $R^2 = 0.993$)

| Main Groups | Subgroups | Injections | Number of Mice |
|-------------|-----------|------------|----------------|
| Control     | Healthy   | -          | 16             |
|             | Saline    | 0.2 mL 0.9% NaCl* | 6 |
|             | Fluconazole | 2 mg/kg fluconazole* | 6 |
|             | Fluconazole | 4 mg/kg fluconazole* | 6 |
|             | Fluconazole | 10 mg/kg fluconazole* | 6 |
| Infection   | *C. albicans* | $3 \times 10^8$ yeast/ml** | 6 |
| Treatment   | *C. albicans* + fluconazole | $3 \times 10^8$ yeast/ml + 10 mg/kg fluconazole*** | 6 |
| Total       |           |            | 52             |

*Injections were applied every 24 hr for two days. TNF-α levels were measured on the 48th hr.
**TNF-α levels were measured six days after the only yeast suspension was injected.
***On the fourth day of the infection the treatment of fluconazole was started, was applied every 24 hr for two days. On the sixth day of the infection TNF-α levels were measured.
Table 2. TNF-α levels of the experimental groups.

| ODs    | TNF-α levels (pg/ml) | Mean values of TNF-α (pg/ml) |
|--------|----------------------|-------------------------------|
| 2mg/kg | 154 36               | 20 ± 14                       |
| Control Groups | 4mg/kg 108 15  | 15 ± 1.1                      |
| 10mg/kg | 128 24               | 29 ± 4.7                      |
| Treatment Group |                          |                               |
| C. albicans + Fluconazole (10mg/kg) | 1453 645  | 683 ± 35                      |
| 1602 715  |                          |                               |
| 1544 688  |                          |                               |

Figure 1. TNF-α levels of all groups.
All of the TNF-α values were calculated with the help of this equation. Friedman test, Mann-Whitney U test, Kruskal-Wallis Variance Analysis were used with the help of the computer programme, SPSS 9:01. That the p value was smaller than .01 or .05 in the statistical evaluations was considered as a significant difference.

RESULTS
The average of TNF-α levels of all groups is shown in the Figure 1. The average of TNF-α level was measured to be 20 pg/ml in the group given 2 mg/kg fluconazole, 15 pg/ml in the group given 4 mg/kg fluconazole, and 29 pg/ml in the group given 10 mg/kg fluconazole. A statistically significant difference was not found among the three groups that were given different doses of fluconazole (p > .05).

The average of TNF-α level was <3 pg/ml in the healthy control group and also in the saline control group which was used as a solvent of fluconazole. There was not a statistically significant difference among the healthy control, saline control, and fluconazole control groups (p > .05).

Candida colonies were identified from the cultures of the kidneys. Mean of the colonies was 28 ± 1.5 of the cultures. The average of TNF-α level was measured to be 683 pg/ml in the treatment group. It was 699 pg/ml in the C. albicans infected group that was left without treatment. There was no statistically significant difference between the two groups (p > .05).

DISCUSSION
Today the treatment of Candida infection is limited to the use of antifungal agents. After infection occurs antifungal agents can be used to directly destroy the causative agent of the infection or it can be used as a non-specific profilactic agent against colonization before the infection. Although Amp B is considered to be the most favorable antifungal to oppose the life-threatening infections, recently non-toxic azole antifungal agents have begun to be preferred to Amp B due to the latter's side effects. Fluconazole is a preperation that can be used systemically and orally. The efficiency of fluconazole has decreased because of its wide use since the beginning of 1990s. Despite the resistance problem, fluconazole still continues to be used all over the world [3].

In our study we investigated if varying doses of systemically administrated fluconazole increased TNF-α or not. As was said in the result section, there was not a statistically significant difference among the three groups to which different doses of fluconazole were applied. That there was not a statistically significant difference between the healthy control group and the three fluconazole control groups made us believe that fluconazole by itself did not have an augmentative effect on TNF-α level. According to our results, it was thought that during the infection, fluconazole did not have an additional effect on TNF-α levels, independent of the effect of C. albicans. In an in vitro study on the subject it was found that there was not a statistically significant difference between TNF-α levels of the peritoneal macrophages cultures of mice incubated with C. albicans plus fluconazole and the TNF-α levels of the cultures incubated solely with C. albicans [4]. In the study of Yamaguchi et al., the immunomodulating activity of antifungal drugs was reviewed. Fluconazole, which has no immunologic effect, did not induce in vitro TNF-α production by macrophages, whereas Amp B slightly but substantially does so [5]. Pitzurra et al. have shown that treatment with fluconazole induced an effect on macrophages similar to that of promoters of TNF-α synthesis and secretion [6]. In the study of Baltch et al., it was evaluated that the effects of cytokines, used singly and in combination, on the microbicidal activity of human monocyte-derived macrophages against intracellular C. albicans in the presence and absence of fluconazole. Their
results demonstrate that in the presence of low concentrations of fluconazole, selected cytokines and their combinations significantly increase the microbicidal activity of human-monocyte derived macrophages against intracellular *C. albicans* [7]. In another study it was demonstrated that fluconazole did not increase TNF-\(\alpha\) level in cerebrospinal fluid of the patients with coccidioidal meningitis [11].

As a result, fluconazole, which was demonstrated to be ineffective on TNF-\(\alpha\) level, was emphasized once more as a safe antifungal agent.

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