

## A Comparison of the Efficacy of Nystatin and Fluconazole Incorporated into Tissue Conditioner on the In Vitro Attachment and Colonization of *Candida Albicans*

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### ABSTRACT

**Background:** Denture stomatitis is a common oral lesion following the use of ill-fitting dentures. A layer of tissue conditioner is usually used to improve adaptation of the denture. These liners can support the *in vivo* adhesion and colonization of the oral *Candida*. The aim of this study was to evaluate the efficacy of the two common antifungal agents mixed with tissue conditioner against *Candida albicans*.

**Methods:** Tissue conditioner disks (Acrosoft) with 5mm diameter and 1mm thickness containing different concentrations of nystatin and fluconazole (1%, 3%, 5%, 10% wt/wt) as well as disks with no antifungal agents (8 disks for each group) were prepared for experimental biofilm formation by inoculation with *Candida albicans* cell suspensions. The specimens were incubated in cell culture microtiter plate wells containing Sabouraud's broth in a rotator shaker at 30°C for 48 hours. Then, the specimens were rinsed and sonicated in sterile water to remove surface organisms. The attached yeasts were enumerated by inoculation of the yeast suspension on Sabouraud's agar. The data was compared using Kruskal-Wallis and Dunn's tests using prism software. P value less than 0.05 was considered significant.

**Results:** The 1% to 10% mixture of nystatin and tissue conditioner completely inhibited the attachment and colonization of *Candida albicans*, although for fluconazole only a 10% concentration caused complete inhibition. Nystatin showed a potentially higher effect in inhibition of *candida* attachment and colonization ( $P = 0.0001$ ) compared to that of fluconazole and a statistically significant difference was seen between 5% and 1% fluconazole ( $P = 0.0001$ ).

**Conclusion:** Tissue conditioner with 1% to 10% nystatin or 10% fluconazole can completely inhibit the adhesion and colonization of *Candida albicans*.

**Keywords:** Candida, Fluconazole, Nystatin, Tissue conditioner.

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### Introduction

Denture stomatitis (DS) is a chronic inflammatory condition of the palatal and alveolar mucosa underlying removable dental prostheses. Denture stomatitis is more commonly seen in the maxillary mucosa. The prevalence of denture stomatitis is varied from 15 to 65%<sup>1</sup> and even more significant in the institutionalized denture wearing population at up to 72%.<sup>2,3</sup> This condition is usually asymptomatic,

although it can be associated with burning, bleeding, an unpleasant taste. Even though several microorganisms can cause this condition, most studies showed that *Candida albicans* and the ill fitting dentures promote the development of this condition. *Candida albicans* is an oral commensally fungus found in 40% of human beings, which facilitates the formation of denture plaque, in

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which *Candida albicans*<sup>4-7</sup> is commonly isolated as the pathogenic agents. The adhesion of *Candida albicans* to denture base material<sup>8-10</sup> particularly when worn continuously in the conditions of trauma and poor oral hygiene can usually cause denture stomatitis.<sup>9</sup> One of the initial steps in prevention and treatment of denture stomatitis is improving denture adaptation and preparing the conditions for recovering of denture bearing tissues. Although tissue conditioners can improve the adaptation of the denture,<sup>10</sup> they are susceptible to the attachment and colonization of oral microorganisms<sup>11,12</sup> especially *Candida* and resulted in the irritation of underlying tissues.<sup>13</sup> Formation of *Candida* biofilm is the result of *Candida* colonization on tissue conditioner surfaces in an aqueous environment<sup>14</sup> following a sequence of events including microbial surface attachment, cell proliferation, matrix production and detachment.<sup>15</sup> Therefore, the prevention of biofilm formation is important for controlling denture stomatitis in denture wearers. Since the use of antifungal agents for prevention and treatment of denture stomatitis caused undesirable taste and also needed frequency doses; it was frequently associated with poor patient compliance.<sup>13</sup> Several studies have attempted to incorporate antifungal agents to tissue conditioner for prevention of plaque formation and treatment of denture stomatitis.<sup>16,17</sup> The purpose of the current in vitro study was to evaluate the effectiveness of the combination of two common antifungal agents with tissue conditioner to inhibit the attachment and colonization of *Candida albicans*.

## Materials and Methods

### *Candida albicans* inoculation preparation

Clinical isolates of *Candida albicans* (ATCC 10231), obtained from the Department of molecular microbiology in Manchester, UK, were used as test organisms for the current experimental study. *Candida albicans* was cultured onto Sabouraud dextrose agar (Oxoid, UK) plate and incubated at 37°C for 3 days. A colony from the stock culture was then diluted in 2 ml sterile saline and a suspension of  $1 \times 10^6$  CFU/ml was prepared. Viable counts were performed to confirm the inoculum size. The number of colonies that appeared after incubation was expressed as CFUs (Colony-Forming Units) and was enumerated with a Haemocytometer slide. The solution was used for further inoculum concentrations.

### Incorporation of antifungals into tissue conditioner

Tissue conditioner, (Acropars, Tehran, Iran) was mixed and prepared according to manufacturer's instruction. Antifungal agents, nystatin (Iran Darou, Iran) and fluconazole (Pars Darou, Iran) were mixed into tissue conditioner powder at concentrations of 1, 3, 5, 10% wt/wt in a sterile plate. A sterile glass rod was used to prepare a thin film of tissue conditioner with 1mm thickness and punched as 5mm diameter disks. (Figure 1) (N = 8). Eight specimens of pure tissue conditioner were also prepared as negative control.



**Figure 1.** Tissue conditioner disks preparation for in vitro biofilm formation.

### Susceptibility testing

Broth microdilution testing<sup>18</sup> was performed in 24-well sterile plastic cell culture microtiter plates), with their corresponding covers as follows: Tissue conditioner disks (containing different concentration of antifungal agents and negative pure disks as control group) were immersed in triplicate in microtiter well containing 900 µl Sabouraud broths (Highmedia, Iran). All disks were contaminated with 100 µl of  $1 \times 10^6$  CFU/ml *C. albicans* cell suspension and the cell culture plate were incubated at 35°C on a rotary shaker (150 rpm) for 48 hours. After incubation, the broth was removed with a sterile pipette. The specimens were rinsed 5 times with sterile water to remove the loosely adherent *C. albicans*.

The specimens were placed in sterile test tubes that contained sterile saline and sonicated for 5 minutes to remove surface organisms. Ten-fold serial dilution of the elution suspension were made, and 100 µl of each elute was placed on duplicate Sabouraud's agar plates. The plates were incubated at 37°C for 48 hours and the colonies were enumerated.

### Statistical tests

The groups were compared by Kruskal-Wallis statistical test. Non-parametric multiple comparative test (Dunn's test) was also used for each test conditions using Prism software. The significant level was set at  $P = 0.05$ .

### Results

Nystatin in all concentrations completely inhibited the attachment and colonization of *C. albicans* ( $P = 0.0001$ ), but in the case of fluconazole, only the 10% wt/wt combination showed complete inhibition of *Candida* colonization while other concentrations reduced the density of *Candida* cells on the tissue conditioner disks. The controls were easily colonized by *C. albicans*. (Table 1)

### Discussion

Most tissue conditioners not only exhibit any antifungal activity, but different studies showed their supporting role in adhesion and growth of *Candida* leading to denture stomatitis in the oral cavity of denture wearers.<sup>11,12</sup> A tissue conditioner with antifungal activity could be a great advantage for patients with a high risk of denture stomatitis.<sup>19</sup> Several attempts have been made to incorporate additives<sup>17,18</sup> and antifungals<sup>16,20,21</sup> to tissue conditioner as a drug delivery method for controlling microbial attachment and colonization.

A number of studies placed agar on top of mixed concentrations of antifungal agents and tissue conditioners to evaluate the efficacy of this mixture by measuring the area of growth inhibition of *C.albicans*.<sup>16,20,22</sup> Different antifungals such as

nystatin, amphotericin B,<sup>23,24</sup> miconazole and ketoconazole<sup>16</sup> combined with different tissue conditioners like Viscogel, Lynal, GC liner and Co comfort have been tested in this manner. Except for Amphotericin B and Viscogel, all other combinations showed a range of growth inhibition results which compared to that of the control group. Addition of herbal medicine to tissue conditioner was also studied for *in vitro* and *in vivo* controlling of denture stomatitis.<sup>18</sup>

However, comparing results from different studies was difficult due to the lack of standardization of concentrations used.<sup>25</sup> Since the addition of antifungal agents into denture liners did not alter the mechanical properties of tissue conditioners,<sup>26,27</sup> in this study the broth dilution method was used to investigate the fungicidal effects of antifungal agents incorporated into tissue conditioners. In this method, *C. albicans* suspensions were prepared from a log phase colony and used for inoculation of the test and control groups. The present method varied from previous studies in that the goal was to record the fungicidal activity more accurately based on the Clinical and Laboratory Standards Institute (CLSI) method rather than the agar assays used in previous studies.<sup>19</sup> Study by Saatchi showed that Acropars tissue conditioner has no antifungal effects by itself.<sup>28</sup>

An oral suspension of both antifungal agents was used in this study (Table 2).<sup>29</sup> Nystatin is the current standard topical treatment for oral candidiasis and fluconazole is the most common systemic antifungal used alternative to topical nystatin.<sup>7</sup>

**Table 1.** *Candida* density (CFU/ml) in the specimens treated with nystatin, fluconazole and in the control group

| Specimens               | Mean | SD   | Median             |
|-------------------------|------|------|--------------------|
| Nystatin [1, 3, 5, 10%] | 0.0  | 0.0  | 0.0 <sup>a</sup>   |
| Fluconazole 10%         | 0.0  | 0.0  | 0.0 <sup>a</sup>   |
| Fluconazole 5%          | 7.1  | 5.2  | 7.3 <sup>b</sup>   |
| Fluconazole 3%          | 15.1 | 11.6 | 18.2 <sup>bc</sup> |
| Fluconazole 1%          | 52.1 | 23.4 | 49.8 <sup>cd</sup> |
| Control                 | 79.2 | 38.1 | 92.3 <sup>d</sup>  |

CFU: Colony forming unit

The groups with identical superscript letter had no significant difference. (Kruskal-Wallis, Dunn's test,  $P = 0.0001$ )

**Table 2.** Recommended dosage of antifungal agents<sup>29</sup>

| Antifungal Agent | Recommended Dosage                      | Equivalence in this study |
|------------------|---|---------------------------|
| Nystatin         | 100, 0000U, 4-5 times/day               | 5% wt/wt                  |
| Fluconazole      | 200 mg [1st day], 100 mg once/day after | 3% wt/wt                  |

In the present study nystatin showed higher inhibitory effects than fluconazole as in all tested combinations completely inhibited the production of *C. albicans* in tissue conditioner disks, however only 10% fluconazole could completely prevent the growth and adhesion of *Candida*. Combination of 3% and 5% fluconazole to Acrosoft were also showed to have significant inhibitory properties compared to control. Chow et al.<sup>25</sup> incorporated nystatin, fluconazole and itraconazole into tissue conditioners to investigate their drug delivery effectiveness using conventional agar based method by measuring their inhibition activities. They showed that itraconazole and fluconazole have more inhibitory activity than nystatin, which is different from the results of our study. The broth microdilution technique used this study was economical and less time-consuming than agar based methods and was able to eliminate uncontrolled results as it facilitated data analysis.<sup>19</sup>

## Conclusion

Incorporation of nystatin rather than fluconazole into tissue conditioners is more effective in treatment of chronic atrophic candidiasis in denture users. Combination of 1% wt/wt nystatin with Acrosoft is recommended for treating candidal infections under dentures.

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