

In- vitro Study of the Effect of Clotrimazole Incorporation into Silicone Soft Liner on Fungal Colonization

Vojdani M. ^{a*}, Zibaei M. ^b, Khaledi AAR. ^c, Zomorodian K. ^b, Ranjbar MA. ^d, Boshehri S. ^d

^a Dept. of Prosthodontic, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

^b Dept. of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

^c Postgraduate Student, Dept. of Prosthodontic, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

^d Dentist, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

KEY WORDS

Denture liner;
C. Albicans;
Clotrimazole.

ABSTRACT

Statement of Problem: Candidal colonization on soft liners is important in the development of pathogenesis, such as denture stomatitis. It has been reported that combining anti-fungal agents into soft liners might be used in treatment and prevention of denture stomatitis.

Purpose: The aim of this *in-vitro* study was to determine whether incorporating clotrimazole (C) into the silicone soft liner (S), would inhibit the growth of *C. albicans* when the specimens are stored in distilled water and washed daily with wet cotton.

Methods and Materials: Experimental specimen disks incorporated with clotrimazole (Sc¹, Sc³⁰, Sc⁶⁰) and without clotrimazole (S¹, S³⁰, S⁶⁰) into the soft liner (no=8) were fabricated aseptically against the polyester film to produce a smooth surface. The treated and control disks were stored in distilled water for 1, 30 and 60 days and washed daily with wet cotton. For fungal growth assessment, they were inoculated with *C.albicans* suspension. The disks were rinsed and sonicated in sterile water to remove surface organisms. The attached yeast was measured by inoculation of the yeast suspension on Sabouraud's agar. The data were compared using two-way ANOVA.

Results: The mean colony forming units (CFU) per mm² for the specimens without clotrimazole after water storage and washing with wet cotton for 1, 30 and 60 days was 6.5×10^6 , 5.8×10^6 and 6.1×10^6 , respectively. CFU for specimens with clotrimazole decreased significantly to 2.6×10^6 and 3.9×10^6 and 4.6×10^6 after 1, 30, and 60 days, respectively. In comparison to those of the control disks, clotrimazole in treated disks was effective in inhibiting *C.albicans* growth significantly following storage in water for 2 months ($P<0.05$).

Conclusion: The addition of clotrimazole significantly reduced *C.albicans* growth to the surface of the silicone soft liner. The samples continued to inhibit the fungal growth when they were washed daily with wet cotton for 2 months.

Received Oct. 2008;

Received in revised form Jan. 2009;

Accepted Nov 2008

* **Corresponding author.** Vojdani M., Address: Dept. of Prosthodontic, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran. Tel: 0711-6240232; Fax: 0711-6270325; E-mail: vojdanim@sums.ac.ir

Introduction

Acrylic or silicone soft liners act as shock absorber and are mostly used as a therapeutic measure for patients who cannot tolerate stresses induced by dentures. These materials are often used in management

of edentulous patients who suffer from chronic pain, traumatized oral mucosa due to prolonged contact between the rigid denture base materials and the underlying tissues. However, despite their vast

clinical benefits, the most challenging factor in the use of long- and short-term soft liners is their tendency to support the growth of *C.albicans* due to material porosity, water absorption and diffusion of nutrient materials. This is further complicated by the difficulty of cleaning most of the liners with routine mechanical or chemical methods [1, 2]. Therefore, prevention of the growth of *C.albicans* has focused on the use of antifungal medications. But topical agents are frequently associated with poor patient cooperation due to unpleasant taste and frequency of dosage. Furthermore, topical therapies are effective on the fungi that invade superficial tissues [3, 4].

Systemic therapy has been reserved for oral infections that are unresponsive to topical agents due to the incidence of serious side-effects [5]. Grüber *et al.* showed that the silicone and methacrylate soft denture liners would support the growth of *C.albicans* [6]. Douglas and Walker incorporated nystatin into a tissue conditioner. Favorable results and also prolonged action of the drug were obtained both *in-vivo* and *in-vitro* [7]. Subsequent studies supported those *in-vitro* results with nystatin [8], as well as miconazole and ketoconazole [9]. Schneid showed that chlorhexidine, clotrimazole, fluconazole and nystatin can be released from the tissue conditioner matrix, demonstrating *in-vitro* growth inhibition of *C.albicans* by these agents. This study has also proved that these four antifungal drugs do not alter the mechanical and physical properties of the tissue conditioner beyond acceptable limits [10].

In a continuous trial to prevent fungal growth on the soft liners, triclosan was added to denture liner. The addition of triclosan did not prevent the growth of *C.albicans* [11]. Nikawa *et al.* suggested that an antimicrobial zeolite combined with soft lining materials would be a potential aid to improve the oral

environment of denture stomatitis patients [12]. Although the results of most studies have been encouraging, it seems that few studies have been conducted on soft liners based on silicone materials.

The purpose of this *in-vitro* study was to determine the effect of the antifungal action of clotrimazole when incorporated into a silicone soft liner.

Materials and Methods

Specimen fabrication

The silicone soft liner selected was GC Reline Extra Soft (G-C Dental Industrial Corp, Tokyo, Japan). As displayed in Table 1, the study was carried out with 6 test groups of S¹, S³⁰, S⁶⁰, Sc¹, Sc³⁰, and Sc⁶⁰ (no= 8 each). The specimens in Group S consisted of GC-Extra Soft without clotrimazole addition. The soft liners were processed according to the manufacturer's direction. The soft liner was conveniently supplied in automix cartridges for ease of dispensing and mixing. The soft lining material was injected directly into the ring molds. Therefore, the specimens were prepared to a uniform size (5 mm in diameter and 1.0 mm thickness) with smooth surfaces by placing polyester film over them. To prepare Sc specimens, clotrimazole powder (Spectrum Chemical Mfg. Corp., Gardena, Calif., USA) was incorporated into silicon samples at 1.0% concentration by weight, and processed as described above. Totally, 48 specimens were fabricated simultaneously. The specimens were allowed to autopolymerize at room temperature for 20 minutes. The specimens (S¹, Sc¹), (S³⁰, Sc³⁰) and (S⁶⁰, Sc⁶⁰) were stored in distilled water at room temperature for 1, 30, and 60 days, respectively and washed very gently by wet cotton for 1 minute each day.

Table 1 Experimental groups of Silicone Soft liner (no=8)

Groups	Conditions
S ¹	Without clotrimazole, stored in distilled water and washed with wet cotton for 1 day
Sc ¹	With clotrimazole, stored in distilled water and washed with wet cotton for 1 day.
S ³⁰	Without clotrimazole, stored in distilled water and washed with wet cotton for 30 days.
Sc ³⁰	With clotrimazole, stored in distilled water and washed with wet cotton for 30 days.
S ⁶⁰	Without clotrimazole, stored in distilled water and washed with wet cotton for 60 days.
Sc ⁶⁰	With clotrimazole, stored in distilled water and washed with wet cotton for 60 days.

Assessment of surface fungal growth

A lyophilized culture of *C.albicans* from a reference laboratory was obtained from the American type culture collection (ATCC 10231). Following the procedure described by Lefebvre *et al* [11], five milliliters of Sabouraud's broth (Stomastat™, Sankin Ind. Co. Ltd, Osaka, Japan) was placed in each test tube and autoclaved. Twenty-four hours before placing the disks, the broth was inoculated with a standard microbiologic loop-full of *C.albicans* to ensure that the organisms were in the active phase of growth when the broth was added to the disks. After 24 hours, the disks were placed on the membrane in a well of a Transwell 12-well plate (Difco, Detroit, MI, USA), with 2 disks per well.

An inoculum of 10^7 CFU / ml was prepared. The adjusted yeast suspension in Sabouraud Dexterous Broth (SDB) was inoculated into each well. The plates were incubated at 37°C for 24h. Growth controls consisting of 1ml of SDB were inoculated for each test. After incubation, the broth was removed with a sterile pipette. The disks were rinsed 5 times with sterile water to remove the loosely adherent *C.albicans*.

The disks were placed in sterile test tubes that contained sterile saline and sonicated for 5 minutes to remove surface organisms. Serial dilution (10x) was made of the eluate, and 100 µl of each eluate was placed on duplicate plates that contained Sabouraud's agar. The plates were incubated at 37° C for 24 hours and the colonies were enumerated. Fungal growth assay was carried out on specimens (S¹, Sc¹), (S³⁰,

Sc³⁰) and (S⁶⁰, Sc⁶⁰) after 1,30 and 60 days, respectively. The data were statistically analyzed in SPSS software (Version 13, Chicago, IL, USA), using two-way ANOVA. The mean values were compared, using the Tukey(HSD) test. $P < 0.05$ was considered significance.

Results

Table 2 shows the *Candida* density (number of colonies/mm²) in both clotrimazole treated disks and untreated controls. A two-way ANOVA was used to find out the differences among the groups (Table 3).

Untreated control Soft Liner was colonized easily by *C.albicans*. The mean colony forming units/mm² for control groups S¹, S³⁰ and S⁶⁰ was 6.5×10^6 , 5.8×10^6 and 6.1×10^6 , respectively. No significant differences were seen in CFU of control disks following storage in distilled water up to 60 days. Lower colonization rate was seen in clotrimazole treated disks in comparison to untreated ones ($P < 0.05$). A significant inhibition of growth was continued at 1, 30 and 60 days storage of experimental specimens in distilled water and washing them daily with wet cotton for 1 minute. The mean colony forming units/mm² for the treated groups Sc¹, Sc³⁰ and Sc⁶⁰ was 2.6×10^6 , 3.9×10^6 and 4.6×10^6 , respectively. Although there was a decrease in the antifungal efficacy of clotrimazole compared with the initial test, there were significant differences among the control and treated specimens at each time interval ($P < 0.05$).

Table 2 Mean, standard deviation, confidence interval and *P-value*, of colony forming per mm² $\times 10^6$

Specimens	N	Mean	Std. Deviation	95% Confidence Interval		P-Value
				Lower Bound	Upper Bound	
S ¹	8	6.5	4.5	6.2	6.9	0.008
Sc ¹	8	2.6	4.7	5.4	6.3	
S ³⁰	8	5.8	5.3	5.7	6.6	
Sc ³⁰	8	3.9	2.7	2.2	2.9	0.008
S ⁶⁰	8	6.1	5.5	3.6	4.1	
Sc ⁶⁰	8	4.6	2.9	4.4	4.9	
Total	48	4.9	1.4	4.5	5.4	

Table 3 Results of two-way ANOVA test

Source	Type III Sum of Squares	Df	Mean Square	F	P-Value
Model	1282.77	6.00	213.79	842.94	0.00
Material	74.00	1.00	74.00	291.78	0.00
Days	5.43	2.00	2.72	10.71	0.00
Material *days	13.31	2.00	6.66	26.24	0.00
Error	10.56	42.00	0.25		
Total	1293.42	48.00			

Discussion

Management of sore or atrophied mucosa, bony undercuts, bruxism and dentures opposing the natural teeth requires the use of resilient lining materials to assist in providing an even distribution of stress on the denture bearing tissues [13-15]. However, it has been shown that the main reservoir of *C.albicans* and related *Candida* species are the fitting surface of the denture and soft lining materials. Resilient liners are easily colonized and deeply infected by these organisms, which may cause oral pathologic condition known as denture stomatitis [1,14]. Clinically, it has been pointed out that continuous swallowing or aspiration of microorganisms from unhygienic dentures exposes patients, particularly the immunocompromised host or medicated elderly, to the risk of unexpected infections [15]. Although routine methods commonly used for denture cleaning include use of immersion cleansers [16] and brushing [17], it has been known that the daily use of these methods can badly affect the physical properties of soft liners [16,17].

Hence, to develop the antifungal soft lining materials as a useful aid for routine plaque control, the present study examined the effect on *C.albicans* growth of clotrimazole incorporation into GC-Extra Soft liner. We found that clotrimazole treated disks stored in water up to two months still have significant inhibitory effect against *Candida* colonization in comparison to control disks. Therefore, the combination of soft liner and antifungal agents would seem to be a logical therapeutic approach to the prevention and treatment of denture stomatitis. By reducing trauma to the denture-bearing tissue in conjunction with administering antifungal therapy on the organism primarily responsible for oral mycotic infection, two important etiologic factors in denture stomatitis are addressed simultaneously [10,18]. Furthermore, this approach can be easily and cheaply prepared by dentists at the chair side; it has no side effects and is not dependent on the patient's compliance [8].

The results of the present study indicated that there was a significant difference in fungal growth on the silicone soft specimens with or without clotrimazole. The antifungal effect of the clotrimazole

specimens decreased after storage in water and washing them with wet cotton for 30 or 60 days, but this reduction was not significant. It is noteworthy that the specimens of clotrimazole-containing soft liner exhibited an effective fungal growth inhibition, but soft liner alone could be colonized by *C.albicans*. Because of silicone composition, chemistry and polymerization mode, these findings were not totally unexpected. The major drawback of silicones as soft liners is their inability to bond denture base resin properly. Despite this shortcoming, silicone soft liners have a myriad of properties that enhances their clinical preeminence over plasticized acrylics [1,2,19]. Silicone soft liners as opposed to acrylic ones are stable in composition and content. They do not contain plasticizer or soluble material that leach out in water during storage periods; therefore, they retain their softness and elasticity over time [1,20]. On the other hand, clotrimazole, which is a synthetic substituted imidazole derivative [21] as opposed to nystatin, has a very stable chemical structure and is not inactivated by heat, light or acid. Clotrimazole is not water-soluble and does not leach out of the silicone samples into water in a significant amount [22,23]. Therefore, it seems that frequent proper washing will not diminish the amount of clotrimazole added to a silicone soft liner for clinical use.

Similar to the previous studies [6-12], we also found that incorporation of antifungal agents into soft liners inhibits fungal colonization. This finding is also in accordance with Pingo *et al's* study which demonstrated that clotrimazole at 1.0% concentration by weight when incorporated into silicon samples was effective in inhibiting *in-vitro* growth of the fungus for 5 months at room temperature, but the effect of long-term water storage was not tested. Increasing the concentration of the clotrimazole did not increase the degree of fungal inhibition. They recommended that 1.0% concentration by weight should be used in any clinical application of this method, without endangering the mechanical and physical properties of the material [23]. However, there is a need to determine the effect of clotrimazole on cytotoxicity and mechanical properties of the silicone soft liners. It should be borne in mind, however, that the clinical

situation differs from *in-vitro* procedure and also clotrimazole may be exhausted earlier than expected under the influence of oral fluids and intake of food-stuff. Therefore, a further *in-vivo* research is warranted.

Conclusion

We showed that the addition of clotrimazole to the silicone soft liner effectively inhibits the growth of the *C.albicans in-vitro*. Also, the clotrimazole specimens continued to inhibit the fungal growth for 2 months when stored in distilled water and washed daily with wet cotton, indicating the efficacy of the antifungal action.

References

- [1] Hickey JE, Zarb GA, Bolender CI. Boucher's Prosthodontic Treatment for Edentulous Patients. 12th ed. St. louis: Mosby; 2004. 200-203.
- [2] Qudah S, Harrison A, Huggett R. Soft lining materials in prosthetic dentistry: a review. Int J Prosthodont 1990; 3: 477-483.
- [3] Schäfer-Korting M. Pharmacokinetics of topical and oral antifungals. Mycoses 1989;32 Suppl 2:34-8.
- [4] Budtz-Jørgensen E. Etiology, pathogenesis, therapy, and prophylaxis of oral yeast infections. Acta Odontol Scand 1990; 48: 61-69.
- [5] Epstein JB. Antifungal therapy in oropharyngeal mycotic infections. Oral Surg Oral Med Oral Pathol 1990; 69: 32-41.
- [6] Gruber RG, Lucatarto EM, Molnar EJ. Fungus growth on tissue conditioners and soft denture liners. Rev Dent Liban 1968; 18: 36-43.
- [7] Douglas WH, Walker DM. Nystatin in denture liners--an alternative treatment of denture stomatitis. Br Dent J 1973; 135: 55-59.
- [8] Thomas CJ, Nutt GM. The in-vitro fungicidal properties of Visco-gel, alone and combined with nystatin and amphotericin B. J Oral Rehabil 1978; 5: 167-172.
- [9] Quinn DM. The effectiveness, in-vitro, of miconazole and ketoconazole combined with tissue conditioners in inhibiting the growth of *Candida albicans*. J Oral Rehabil 1985; 12: 177-182.
- [10] Schneid TR. An in vitro analysis of a sustained release system for the treatment of denture stomatitis. Spec Care Dentist 1992; 12: 245-250.
- [11] Lefebvre CA, Wataha JC, Cibirka RM, Schuster GS, Parr GR. Effects of triclosan on the cytotoxicity and fungal growth on a soft denture liner. J Prosthet Dent 2001; 85: 352-356.
- [12] Nikawa H, Yamamoto T, Hamada T, Rahardjo MB, Murata H, Nakanoda S. Antifungal effect of zeolite-incorporated tissue conditioner against *Candida albicans* growth and/or acid production. J Oral Rehabil 1997; 24: 350-357.
- [13] Wright PS. The success and failure of denture soft-lining materials in clinical use. J Dent 1984; 12: 319-327.
- [14] Nikawa H, Yamamoto T, Hamada T. Effect of components of resilient denture-lining materials on the growth, acid production and colonization of *Candida albicans*. J Oral Rehabil 1995; 22: 817-824.
- [15] Nikawa H, Hamada T, Yamamoto T. Denture plaque--past and recent concerns. J Dent 1998; 26: 299-304.
- [16] Nikawa H, Jin C, Makihira S, Egusa H, Hamada T, Kumagai H. Biofilm formation of *Candida albicans* on the surfaces of deteriorated soft denture lining materials caused by denture cleansers in vitro. J Oral Rehabil 2003; 30: 243-250.
- [17] Oliveira LV, Mesquita MF, Henriques GE, Consani RL. The effect of brushing on surface roughness of denture lining materials. J Prosthodont 2007; 16: 179-184.
- [18] Addy M. In vitro studies into the use of denture base and soft liner materials as carriers for drugs in the mouth. J Oral Rehabil 1981; 8: 131-142.
- [19] McCabe JF, Carrick TE, Kamohara H. Adhesive bond strength and compliance for denture soft lining materials. Biomaterials 2002; 23: 1347-1352.
- [20] Mutluay MM, Ruyter IE. Evaluation of bond strength of soft relining materials to denture base polymers. Dent Mater 2007; 23: 1373-1381.
- [21] Sande MA, Mandell GL. Antimicrobial agents: antifungal and antiviral agents. In: Gillman AG, Goodman LS, Rall TW, Murad F, eds: Goodman and Gillman's the pharmacologic basis of therapeutics. 7th ed. New York: Macmillan Publishing Co; 1985. 1219.
- [22] Shadomy S, Pfaller MA. Laboratory studies with antifungal agent susceptibility tests and quantization of body fluids. In: Balows A, Hausler WJ, Herrmann KL, Isenburg HD, Shadomy HJ. Manual of Clinical Microbiology. 5th ed. Washington; DC: American Society for Microbiology: 1991. 1173: 83-88.
- [23] Pigno MA, Goldschmidt MC, Lemon JC. The efficacy of antifungal agents incorporated into a facial prosthetic silicone elastomer. J Prosthet Dent 1994; 71: 295-300.