Comparative effects of two different artificial body fluids on *Candida albicans* adhesion to soft lining materials

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This study investigated the *C. albicans* adhesion to cold- and heat-polymerized soft lining materials that were initially incubated in two different artificial body fluids, namely saliva and nasal secretion, and examined the surface roughness the materials (cold and heat polymerized soft liner) tested *in vitro*. Cold (Visco Gel) and heat-polymerized (Molloplast B) soft liner specimens (*N*=32, *n*=8 per group) (10×10×1.5 mm) were randomly produced to express the relationship between surface roughness and contamination, and influence of body fluids, and incubated in 1.5 ml contaminated solutions for 2 h. After fixation, all of materials were evaluated under optical microscope (×400) and SEM. Surface roughness measurements were examined with profilometre for each material. Data were analyzed using two-way ANOVA, Tukey’s HSD and Dunnett T3 tests (*p*<0.05). Material type (*p*<0.05) and contamination media (*p*<0.05) showed a significant influence on the *C. albicans* adherence. The surface roughness of cold polymerized soft liner (Visco Gel) was significantly higher than heat-polymerized soft liner (Molloplast B) (*p*<0.05).

**Keywords**: Adhesion, *Candida albicans*, Denture soft lining materials

**INTRODUCTION**

Human body, as a host, has many kinds of resident microorganisms that could be classified as both domestic and opportunistic pathogens1. These organisms are closely related with the host systems such as cell-host tissues or cell-biomaterial interactions that could be harmful for the host. Some of the remainder organisms are opportunistic pathogens. The members of the resident microflora spread out in the host area (i.e. mouth, skin, gut)2. One such residential human microflora is *Candida albicans* (*C. albicans*) which is a eukaryotic and opportunistic pathogen, causing candidiasis known as the most common fungal infection in human being3-10. This organism being mainly found in oral cavity causes stomatitis. Denture stomatitis, more commonly known as ‘denture sore mouth’, is frequently observed in the maxilla of elderly denture wearers11-18. Prevalence of denture stomatitis in association with *C. albicans* has been reported to range between 11 and 67% in complete denture wearers19-24. Continuous denture wearing appears to facilitate denture stomatitis by increasing the local injury and the time of mucosal exposure to denture plaque25-30. Resilient denture lining materials are used to overcome such injury. Such materials reduce the traumatic effect that a denture may have on patients with thin atrophic mucosa or with normal mucosa but with resorbed ridges, sharp alveolar ridge crest, deep anatomic undercuts, bony protuberances, bruxomania, or where the oral mucosa exhibits a reduced tolerance to the load applied by the denture31-33. They also facilitate comfort when used in obturators for acquired and congenital cleft palate34,35. Currently, soft lining materials are available in silicone elastomers and soft acrylic compounds. Soft denture liners are also defined as soft polymers which may be applied to the fitting or mucosal surface of the dentures. They reduce the occlusal forces and distribute them more evenly on the underlying mucosal tissues36. These materials can be classified as provisional or definitive according to their composition being either silicone rubber or acrylic resin. Soft denture liners can be either cold (chemically)- or heat-polymerized37,38. The adherence of *C. albicans* to host cells or polymers such as denture acrylic resins or soft lining materials is the first step in colonization, yielding to development of pathogenesis and eventually causing infection39-42. Soft lining materials have been found to be more prone to microbial adhesion than acrylic resin denture base materials43-46. They have demonstrated the ability to interact with oral microorganisms because of their surface texture and the physical and chemical affinity to microorganisms. Verran and Maryan47 showed more fungal adhesion was found on rough surfaces than smooth surfaces. Differences in surface topography affect the attachment of microorganisms to a surface, with higher numbers of cells retained on rougher surfaces. Surface irregularities would increase the likelihood of microorganisms remaining on the surface47. When soft lining materials are used for relining the maxillofacial prosthesis, they may expose to the nasal secretion. The relationship between the salivary or nasal secretions on denture material surfaces and *C. albicans* colonization is complex, particularly when the surface aging of soft lining materials are taken into account48. When wearing a denture, the base becomes colonized with pellicles...
composed of salivary or nasal secretions which may provide receptor sites for the adherence of microorganisms. Some studies have reported that soft lining materials have an inhibitory effect on yeast growth providing that additional variables may contribute to yeast growth in vivo. In fact, since yeast cells tend to colonize more in acidic conditions, it can be hypothesized that nasal secretion, due to its low pH, would result in more microbial colonization. Dental literature contains many case reports on the construction or application silicone liners but to the authors’ knowledge there is no information on C. albicans adhesion in nasal secretion. Particularly in patients who have gone tumor surgery and thereafter acquire obturators for maxillofacial defects, post-nasal secretion is often unavoidable. In fact, especially in patients with postnasal flux, intra-oral prosthetic appliances are also exposed to nasal fluid contamination. In such situations, oral flora of the patients is affected. Hence, prosthesis exposed to such environments may be indirectly affected from the microorganisms in nasal secretion.

The objectives of this study therefore were to investigate C. albicans adhesion and surface roughness onto two commercial silicone liners based on different polymerization processes in the presence of artificial saliva and nasal secretion and to evaluate the colonization microscopically. The tested null hypotheses were that nasal secretion would result in more C. albicans colonization than artificial saliva and heat-polymerization would result in less C. albicans adhesion.

MATERIALS AND METHODS

Two different denture soft lining materials were used in this study, namely Visco Gel (Dentsply Ltd., De Trey Division, Weybridge, U.K), a cold-polymerized transparent acrylic resin soft lining material (Batch#: 071000;1479) and a heat-polymerized one, Molloplast B (Detax Karl Huber GmbH, Ettingen, Germany) (Batch#: 17-610) (Table 1). While cold-polymerized soft liner (Visco Gel) was supplied as a powder, liquid and a sealer liquid with the chemical composition based on polyethylmethacrylate (powder) and phthalyl butyl glycolate and ethanol (liquid), heat-polymerized soft liner (Molloplast B) was supplied as a single paste and adhesive (bonding liquid) based on polydimethylsiloxan (polymer) and benzyl peroxide (initiator).

Specimen preparation
Pink modeling wax forms (10×10 mm, thickness: 1.5 mm) were punched from a sheet of wax. Dental stone (BPB Formula, Newark Works, Bowbridge Lane, Newark, Nottinghamshire, UK) was mixed, poured into the shallow part of a dental flask and allowed to set. After setting, the stone was lubricated using petroleum gel. Consequently, specimens were flasked with vacuum, heated for 10 min, and the wax was removed using a hot water spray. Flasks were cooled, and then the stone was lubricated using a CO-MO sealant (John Winter and Co. Ltd, Halifax, UK). Wet polymerization technique was used for the heat-polymerized liners (Fig. 1). The cold-polymerized soft lining material (Visco Gel) was processed according to the manufacturer’s directions and polymerized in the flask for 15 min at the room temperature, and then made to a uniform size (10×10×1.5 mm thickness) using the mold (Fig. 1). After polymerization, specimens trimmed and kept in polyethylene containers in a dry environment until required (1 week). In order to

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Polymerization mode</th>
<th>Chemical composition</th>
<th>Shade</th>
<th>Batch number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visco Gel</td>
<td>Cold-cured acrylic resin soft lining material</td>
<td>Polyethylmethacrylate (powder) and phthalyl butyl glycolate, ethanol (liquid)</td>
<td>Translucent</td>
<td>071000;1479</td>
<td>Dentsply Ltd., De Trey Division, Weybridge, U.K.</td>
</tr>
<tr>
<td>Molloplast B</td>
<td>Heat-cured silicone soft lining material</td>
<td>Polydimethylsiloxan (polymer) and benzyl peroxide (initiator)</td>
<td>Pink</td>
<td>17-610</td>
<td>Detax Karl Huber GmbH, Ettingen, Germany</td>
</tr>
</tbody>
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Fig. 1 Test specimens with smooth surface (10×10×1.5 mm).
produce test specimens of comparable smoothness, they were prepared against glass.

Surface roughness of soft lining materials
The surface roughness (Ra) of the test samples was measured with a profilometre (Mitutoyo Surf Test 301, Tokyo, Japan), where a stylus traverses across the layer of the surface, and an amplified trace of the profile is provided. The Ra value is the arithmetical average of all departures of the profile through the mean sample length. All measurements were recorded by one operator.

Candida survey
C. albicans strain ATCC 60193 was obtained from microorganism stock culture (Basic and Industrial Microbiology Section, Department of Biology, Ege University, Izmir, Turkey). For enrichment of microorganisms, stock culture was inoculated into Mueller Hinton Broth, Fluka® with 500mM sucrose medium and incubated for 24 h. After incubation, cells were collected by centrifugation (Hettich Rotina 35 R Zentrifugen, Germany), harvested and washed three times in sterile 0.1M phosphate buffered saline (PBS) with 0.89% NaCl at pH: 7.2, and re-suspended in PBS to an optical density of 0.5 at 540 nm, corresponding to a cell concentration of 1-5×10^6 CFU/ml. OD of cell suspension was previously determined in either saliva or nasal secretion for the specimens to be inoculated in saliva or nasal secretion, respectively.

Adherence assay
Specimens were sterilized in an autoclave (HiClave^TM HC-50L, Hirayama, Japan) at 121°C for 20 min. They were then placed in separate sterile tubes and incubated with either 1.5 ml artificial saliva (pH=7) or with 1.5 ml artificial nasal secretion (pH=4.8) at 37°C with orbital shaking (100 rpm) for 2 h. The artificial saliva was composed of 0.220 g/L calcium chloride, 1.07 g/L sodium phosphate, 1.68 g/L sodium bicarbonate, and 2 g/L sodium azide 0.2% (NaN₃<sup>3</sup>) and artificial nasal secretion consisted of Na<sup>+</sup> 107±4 mM, Cl<sup>−</sup> 120±6 mM, K<sup>+</sup> 8.7±0.4 mM obtained according to in vivo microdialysis procedure (IVMD)<sup>35</sup>. After incubation, in order to remove the unattached cells, the specimens were gently removed from the tubes and rinsed by dipping them into the PBS solution for three times for approximately 75 s. Then, for fixation of the attached cells, specimens were treated with 100% ethanol for 3 s and left to dry in sterile plates. Specimens were stained using sterilized, fixated Methylene Blue stain (Merck<sup>4</sup>) for 1 min and subsequently, evaluated under optical light microscope (Olympus CH20, Olympus Singapore PTE Ltd., City, Singapore) at ×400 magnification<sup>4</sup>. On each specimen, 15 different consecutive areas were counted. Visible measurement field was calculated in mm<sup>2</sup> and the obtained data were expressed in cell/mm<sup>2</sup>. Complementary to the optical microscopy analysis, specimens were also observed under Scanning Electron Microscope (SEM) (JEOL JSM-5200, Kyoto, Japan) after they were fixed with 2% gluteraldehyde, dehydrated with ethanol (at 25, 50, 75 and 100% for 5, 5, 5 and 10 min, respectively) and coated with Au-Pd (×750 to ×3500 magnification).

Statistical analysis
The statistical analysis was performed with the SPSS software package (version 11.5; SPSS, Chicago, IL, USA). Mean ranks obtained from adherence assay were evaluated with using two-way analysis of variance (ANOVA) and paired sample T-test. Since significant differences were found between or within groups, Tukey’s HSD was used to determine the differences. The results of normality and homogeneity test (Kolmogorov-Smirnov) indicated that the residual values were normally distributed when plotted against predicted values. The uniformity and normality tests did not violate the statistical assumptions. In all comparisons, statistical significance was declared if the p value was less than 0.05.

**RESULTS**

Surface roughness
The results are presented as surface roughness values (a Ra unit) of average surface roughness measurements of soft lining materials in Table 2. The number of C. albicans adherence to smooth surfaces was low. Significantly more cells were counted on the rough surfaces than on smooth surfaces. The surface roughness of cold-polimerized soft liner (Visco Gel) was significantly higher than heat-polimerized soft liner (Molloplast B) (p<0.05).

C. albicans adherence
Both the material type (p<0.05) and contamination media (p<0.05) showed a significant influence on the C. albicans adherence (two-way ANOVA, paired sample T-test).

Cold-polimerized soft lining (Visco Gel) material

<table>
<thead>
<tr>
<th>Materials</th>
<th>Ra / Mean - (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visco Gel (n=10)</td>
<td>15.67 (2.20)</td>
</tr>
<tr>
<td>Molloplast B (n=10)</td>
<td>0.21 (0.06)</td>
</tr>
</tbody>
</table>

**Table 2 Mean surface roughness values (Ra) and standard deviations for surface roughness**

Ra: A unit of the arithmetical average of all departures of the profile through the mean sample length.
SD: Standard deviation
n: Number of samples
showed more *C. albicans* adherence in both saliva and nasal secretion (mean rank: 9040.75 and 2550.55, respectively) than that of heat-polimerized soft liner (Molloplast B) (mean rank: 4149.18 and 1350.31, respectively) \((p<0.05, \text{Tukey’s HSD})\).

*C. albicans* adherence onto cold-polimerized soft liner (Visco Gel) specimens that were incubated with nasal secretion (mean rank: 3900.86) was significantly less than with saliva (mean rank: 13189.93) \((p=0.05)\). Approximately 3.5 fold difference was observed between nasal and saliva in adhesion results (Fig. 2).

**Microscopical evaluations**

In general, *C. albicans* adherence was observed in cluster forms on all of materials. Whole attached cells were viewed in blastospore morphology. While cold-polimerized soft liner (Visco Gel) specimens presented local colonization of the cells, heat-polimerized soft liner (Molloplast B) specimens exhibited smoothly dispersed cells (Figs. 3a-c).

**DISCUSSION**

Diseases or inflammations such as stomatitis may occur as a consequence of accumulation ability of the cells on the polymeric materials that is closely related to the London- van der Waals (the surface energy between particles which generates dipole interactions) and electrostatic forces that facilitate cell adhesion\(^{39,40}\). When the surface roughness is increased, it triggers microbial adhesion and the number of adhered cells also increases remarkably\(^{4,27,39}\). During microbial colonization, cells produce acidic substances as an outcome of their natural metabolisms that affect the pH of the surface they are interacting with. On the other hand, pH of the medium to which the materials are exposed plays also a significant role on microbial colonization\(^{41}\). This study was undertaken in order to investigate the *C. albicans* adherence levels to commercial denture soft liners when they were exposed to two different human body secretions, namely saliva and nasal secretions that were artificially created. The high number of yeast cells presented as “per mm\(^2\)” facilitated comparison with other studies, but is somewhat misleading because three-dimensional accumulations of cells, not monolayers, were observed and counted. Regardless of the polymerization type of the materials tested, increased *C. albicans* adherence was found in artificial saliva (pH=7). Although artificial saliva was prepared at neutral state, by-products of *C. albicans* could have changed the pH of the whole solution\(^{42,43}\). Artificial saliva can be served as a screening medium in this study.

Contamination of the specimens with artificial nasal secretion yielded to significantly less *C. albicans* adherence compared to artificial saliva. Therefore the hypothesis is rejected. The nasal secretion prepared had a pH of 4.8. Less *C. albicans* adherence in this medium could be attributed to the presence of NaNO\(_3\), in this medium that might have inhibited colonization, created ionic bonds and thereby had repellent effect.
Other reason could be due to the storage duration of the specimens in this media (2 h) which may not be the critical time point. Unfortunately, to the authors’ knowledge, there exists no study in the dental literature using nasal secretion as a contamination medium for soft liners to make comparisons of the findings of this present study. In this study, artificial saliva and nasal secretion were used for all specimens separately. Interaction between organism and fluids that are aforementioned cannot be commented on in this situation. It was reported that in-vivo microorganisms never attach directly to denture materials without salivary pellicle. In this experiment, although no pellicle was initially formed and all specimens were incubated directly with artificial saliva and nasal secretion that was added to *C. albicans*, the results showed that *C. albicans* does not require pellicle for adhesion. Although composition of nasal secretion has been defined earlier, *C. albicans* adherence on soft lining materials in the presence of artificial nasal secretion was not studied. Currently, in a case controlled clinical trial, the effect of human nasal secretion on *C. albicans* adherence on liners is being evaluated where in-vivo results will be collected and correlated to the findings of this in-vitro study. This study therefore should be considered for screening purposes.

In this study, artificial saliva and nasal secretion coated heat (Molloplast B) and cold (Visco Gel)-polymerized soft liners were tested. In all materials tested, some degree of *C. albicans* adherence was noted being significantly less for heat-polymerized soft liner (Molloplast B), confirming the hypothesis. On the other hand, the SEM findings and surface roughness measurement applied with profilometre presented fairly smooth surfaces with heat-polymerized soft liner (Molloplast B) and cold-polymerized soft liner (Visco Gel) that is most probably related to the polymerization modes of the materials tested. According to the ‘Tari et al.’, Nikawa et al., in their investigation on fungal adherence with or without a salivary, showed there was no difference in fungal adherence between to saliva coated and uncoated soft lining materials. Therefore uncoated materials as control material was not tested in this study. Two different soft lining materials were processed against glass slides to the direction of suggestions of researchers’ studies in reviewed literatures. Still, there was a difference in the adhesion of *C. albicans* between soft lining materials was observed. This result essentially promoted that polymerization method was effective. Besides, more adhesion may be observed on the materials when processed against dental stone.

Larger cells such as yeasts are more easily dislodged from smooth surfaces than the smaller microorganisms. In this study, the exposure of the specimens to either artificial saliva or nasal secretion was 2 h due to technical reasons. Although salivary proteins has been considered to serve as a source of nutrients for microorganisms for growth and reproduction requirements, as well as assisting in microbial succession of dissimilar bacterial species in developing plaque, prolonged period of contamination may lead to possible cell death. In fact, prolonged exposure of the soft liners to such media may have more in-vivo implications regarding the functional lifespan of liners since host surfaces in contact would be then more heavily colonized with commencing microorganisms, many of which are opportunist pathogens. The synergistic effect of the microorganism colonization with low pH together with the conditions of the contamination media may lead to material degradation and eventually rougher surfaces.

In this study, to determine of surface texture of the liners, roughness measurements were undertaken. The surface roughness of a material used for a removable prosthesis is a importance since it affects, directly or indirectly, retention, staining resistance, plaque accumulation, as well as oral tissue health and patient comfort. In the present study, cold-polymerized soft liner (Visco Gel) was rougher than heat-polymerized soft liner (Molloplast B). The surface roughness value for heat-polymerized soft liner (Molloplast B) and cold-polymerized soft liner (Visco Gel) was found similar to those reported by Tari et al. This may be because the same technique was used for measurement. Separately, microscopy analysis has shown heat-polymerized soft liner (Molloplast B) with smooth surfaces. Surface roughness is a significant factor in the attachment and adhesion of microorganisms on surfaces with an increase in roughness causing increased retention of cells. It is known to be a factor in the entrapment of microorganisms on surfaces and their protection from shear forces. In this study, yeast cells were retained on roughened surfaces in higher numbers than on smooth. Cells were observed within surface irregularities. The results of present study may be explained by the effect reported previously. Namely, it demonstrated that increased surface roughness increased retention of yeast.

From the clinical point of view, maxillofacial and dental/oral prostheses mounted into patients should be frequently controlled by the dentist. Particularly in patients who have gone tumor surgery and thereafter acquire obturators for maxillofacial defects, post-nasal secretion is often unavoidable. Such prosthesis exposed to the nasal area comprises appliances for nasal, midfacial, large orbital defects, combined facial prosthesis with defects in the maxilla and tracheal stent. In fact, especially in patients with postnasal flux, intra-oral prosthetic appliances are also exposed to nasal fluid contamination. In such situations, oral flora of the patients is affected. Hence, prosthesis exposed to such environments may be indirectly affected from the microorganisms in nasal secretion. Based on the obtained data, maxillofacial and oral/dental prostheses exposed to nasal secretion seems to collect less *C. albicans* than the ones exposed to saliva. Nonetheless, smooth surfaces and heat-polymerization of such materials may delay microorganism colonization. In
this study, *C. albicans* was used as a model microorganism. The obtained results may change with other *Candida* species such as *C. tropicalis*, *Candida dubliniensis*, *Candida glabrata*, *C parapsilosis*, *C. guilliermondii*, or with other microorganisms such as *S. aureus* or *S. mutans*. In this study, no microbial agents were used in the “cleaning” (washing) process, but it would be of interest to compare the effectiveness of denture cleansing agents against microorganisms immobilized on surfaces.

CONCLUSIONS

Cold-polymerized soft-lining material tested showed more *C. albicans* adherence in both saliva and nasal secretion being widely spread onto the material than that of the heat-polymerized one, where *C. albicans* accumulation was observed more concentrated in blastospore morphology. The surface roughness of cold-polymerized soft liner (Visco Gel) was significantly higher than heat-polymerized soft liner (Molloplas B). Cold-polymerized soft liner (Visco Gel) with roughened surface showed a greater adherence of *C. albicans*. Contamination of the soft liners in artificial nasal secretion led to less *C. albicans* adherence than that of artificial saliva.

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