Cytotoxicity of hard and soft denture lining materials

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The cytotoxicity of nine soft and hard lining materials (Mollosil Plus, Ufi Gel SC, Visco-gel, Molloplast-B, GC Tissue Conditioner, Vertex Rapid Simplified, GC Reline Hard, Vertex Self-Curing, Ufi Gel hard C) was evaluated using human gingival fibroblasts (HGFs). Twelve disk samples per lining material were prepared and incubated for 24, 48, 72, and 96 h. Cytotoxicity of each lining material’s extract on cultured HGFs was measured using XTT assay. Data were analyzed using one-way ANOVA, post hoc Dunnett’s T3 and Bonferroni tests at a significance level of \( p<0.05 \). At all incubation periods, all the hard lining materials (Vertex-SC, GC Reline Hard, Vertex-RS, and Ufi Gel hard C) showed cell viability higher than 90%. Among the soft lining materials, although there were no significant differences in cell viability among the different incubation periods for each lining material \( (p>0.05) \), autopolymerized acrylic-based GC Tissue Conditioner showed significantly lower cell viability than the other soft lining materials at each incubation period. Among the hard lining materials, there were no significant differences both among the materials and across all incubation periods for each lining material \( (p>0.05) \). In conclusion, all soft and hard liners exhibited good biocompatibility regardless of incubation time, except for GC Tissue Conditioner.

Keywords: Cytotoxicity, Human gingival fibroblasts, Soft lining materials, Hard lining materials

INTRODUCTION

Hard and soft denture lining materials are polymers used on the intaglio surfaces of dentures to increase adaptation of a denture to the oral tissues. Denture liners are in direct contact with oral mucosa, which means they must be non-irritating, non-toxic, and inhibit bacterial and fungal colonization¹.²

Denture lining materials are susceptible to changes during functioning in the oral cavity. Denture liners may contain ingredients that can leach out. Consequently, loss of ethanol or plasticizer, or deterioration of the material itself, causes substances to be absorbed into the material. These leaching and absorption incidents result in a spectrum of mechanical, physical, and biological changes in the properties of denture lining materials simultaneously or over time³, such as volumetric change, hardening, color change, and cytotoxicity change. Leached cytotoxic components cause direct irritation of mucosa, and large areas of oral mucosa are exposed to these irritating or toxic compounds over an extended period⁴.⁵

Apart from evaluation of physical properties, biocompatibility evaluation of a dental material is a prerequisite before it is accepted for clinical use⁶. Cell culture studies are a very sensitive method that investigates toxicity in a simplified system so as to minimize the effect of confounding variables⁷. As the first step in the biocompatibility evaluation of dental materials, cell culture methods are widely endorsed as a relatively simple, reproducible, and cost-effective technique which can be carefully controlled⁸.

Various cell types can be used to quantitatively investigate the cytotoxicity of dental materials and to evaluate different biological endpoints⁹. Given that gingival tissue is in immediate, direct contact with hard and soft lining materials, it is important to clarify the cytotoxic effects of these agents on fibroblast cells derived from human gingiva.

XTT (sodium 3’-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) cell proliferation assay is a colorimetric assay system which was first described in 1988 by Scudiero et al. as an effective method to measure cell growth and drug sensitivity in tumor cell lines¹¹.¹² Metabolically active cells reduce slightly yellow tetrazolium salt XTT to a bright orange formazan product. The formazan product of XTT reduction is soluble and can be used in real-time assays since it can be directly quantified using an ELISA reader. XTT assay has many advantages: easy to use with no need for solubilization step, high throughput with fast results, high sensitivity using low cell numbers, and reproducible accuracy. In addition, this assay is non-radioactive and is thus preferred over cytotoxicity tests based on \( ^{51}\text{Cr} \) release from cells¹³.

In this \textit{in vitro} study, the aim was to evaluate the cytotoxicity of nine commercially available soft and hard denture lining materials using cell culture method with human gingival fibroblasts (HGFs). Cytotoxicity was then measured using XTT cell viability assay.

MATERIALS AND METHODS

The study protocol of this study was approved by the Ethics Committee of Ege University (Number: 10-6/13, Date: 18 June 2010). All patients gave their signed informed consent.
consent prior to collection of gingival tissue samples.

Medium extracts of denture lining materials
Nine different soft and hard lining materials were tested in this study, and their details are presented in Table 1. For each of the nine lining materials, 12 disk-shaped samples ($\varnothing=16$ mm, $h=1.5$ mm) were prepared according to the guidelines of ISO 10993: Tests for Cytotoxicity —In Vitro Methods. A total of 108 samples ($n=12$ per denture lining material) were thus obtained.

Disk samples were exposed to 16 kGy gamma irradiation sterilization (Gamma-Pak Sterilization Ind., Tekirdag, Turkey). Sterilized samples were placed in 24-well plates and submerged in 500 µL/well of Dulbecco’s modified Eagle’s medium (DMEM; Biological Industries, Kibbutz Beit Haemek, Israel) containing 10% fetal calf serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin to obtain medium extracts of the lining materials. All 24-well plates were incubated in a highly humidified atmosphere containing 5% CO$_2$ at 37°C. After 24 h, the medium was removed from each well. Samples were rinsed with 500 µL of PBS (Phosphate-Buffered Saline) and submerged again in 500 µL of DMEM. Collected medium extracts were kept in sterile Eppendorf tubes at $-20^\circ$C until cytotoxicity experiments. This procedure for 24-h incubation was repeated for all medium extracts collected after 48, 72, and 96 h of incubation.

Cell cultures
Human gingival fibroblasts (HGFs) were derived from three biopsies of healthy gingival tissues obtained during surgical removal of impacted third molar teeth in three patients. Each biopsy sample was placed in DMEM culture medium for transport to the laboratory.

Gingival tissue biopsy samples were washed in DMEM and cultured in tissue culture flasks using the explant culture method. HGFs were grown in a highly humidified atmosphere containing 5% CO$_2$ at 37°C. Culture medium was supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Media were changed every 3 days, and cells were passaged when confluent with 0.05% trypsin in 0.02% ethylenediaminetetraacetic acid (Biological Industries, Kibbutz Beit Haemek, Israel). After trypsinization, cells were re-plated at a density of $3\times10^4$ cells/cm$^2$. To minimize differences in subcultures, cells were subcultured until passage 10.

Cytotoxicity assay
Cytotoxicity was evaluated using XTT Cell Proliferation Kit (Roche Applied Science, Basel, Switzerland). HGF cells were plated at $1\times10^5$ cells/well in a 96-well plate and incubated for 24 h. After the culture medium was aspirated, 100 µL of medium extract of each denture lining material was pipetted immediately into each well containing HGF cells. Each assay was run in triplicate. Formazan formation was quantified spectrophotometrically at 450 nm using a microplate reader (Thermo, Vantaa, Finland) after 24, 48, 72, and 96 h of incubation. Cell viability was calculated using the formula below$^{14}$:

\[
\text{Cell viability} = \frac{\text{Formazan formation of sample}}{\text{Formazan formation of control}} \times 100
\]

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
<th>Batch No.</th>
<th>Material type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mollosil Plus</td>
<td>Detax, Germany</td>
<td>0907605</td>
<td>Autopolymerized, silicone-based, permanent, soft lining material</td>
</tr>
<tr>
<td>Ufi Gel SC</td>
<td>Voco, Germany</td>
<td>0921267</td>
<td>Autopolymerized, silicone-based (A-silicone), permanent, soft lining material</td>
</tr>
<tr>
<td>Visco-gel</td>
<td>Dentsply, UK</td>
<td>0710001479</td>
<td>Autopolymerized, acrylic-based, temporary, soft lining material</td>
</tr>
<tr>
<td>Molloplast B</td>
<td>Detax, Germany</td>
<td>090103</td>
<td>Heat-polymerized, silicone-based, permanent, soft lining material</td>
</tr>
<tr>
<td>GC Tissue Conditioner</td>
<td>GC, Japan</td>
<td>0902061</td>
<td>Autopolymerized, acrylic-based, temporary, soft lining material</td>
</tr>
<tr>
<td>Vertex Rapid Simplified</td>
<td>Vertex-Dental, Netherlands</td>
<td>YH412P01</td>
<td>Heat-polymerized, methyl methacrylate-based, conventional acrylic resin</td>
</tr>
<tr>
<td>(Vertex-RS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC Reline Hard</td>
<td>GC, Japan</td>
<td>0901191</td>
<td>Autopolymerized, acrylic-based, permanent, hard lining material</td>
</tr>
<tr>
<td>Vertex Self-Curing</td>
<td>Vertex-Dental, Netherlands</td>
<td>XY062P05</td>
<td>Autopolymerized, poly(methyl methacrylate)-based, conventional acrylic resin</td>
</tr>
<tr>
<td>(Vertex-SC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ufi Gel hard C</td>
<td>Voco, Germany</td>
<td>0939169</td>
<td>Autopolymerized, acrylic-based, permanent, hard lining material</td>
</tr>
</tbody>
</table>
Cell viability (%) = \[ \text{Mean OD of test group/Mean OD of control group} \times 100\% \]

Cell viability was scored according to the method of Sjogren et al.\textsuperscript{15}. If cell viability exceeded 90\%, the material was deemed non-cytotoxic. For cell viability at 60–90\% range, the material was regarded as slightly cytotoxic. For cell viability at 30–59\% range, the material was regarded as moderately cytotoxic. For cell viability below 30\%, the material was considered severely cytotoxic.

For each denture lining material, a total of 27 of experiments (3 repeated wells×3 primary cell lines×3 independent experiments) were carried out by two operators.

**Statistical analysis**

Results of XTT assay were analyzed by one-way ANOVA using SPSS v.15.0 for Windows (SPSS Inc., Chicago, USA). Differences between test groups were assessed using post hoc Dunnett’s T3 and Bonferroni tests. Statistical significance was set at \( p<0.05 \).

**RESULTS**

Table 2 shows the cell viability results of both soft and hard lining materials after 24, 48, 72, and 96 h of incubation.

**Cytotoxicity assay results of soft denture lining materials**

At 24 h, Visco-gel exhibited the highest cell viability (109.95\%) followed by Mollosil Plus (99.95\%) and Ufi Gel-SC (97.53\%). At 48 h, Ufi Gel-SC exhibited the highest cell viability (105.4\%) followed by Mollosil Plus (103.76\%) and Molloplast-B (98.68\%).

Throughout the entire test period, Visco-gel, Mollosil Plus, and Ufi Gel-SC continued to exhibit cell viability higher than 90\%. Therefore, these materials were deemed as non-cytotoxic. For Molloplast-B, cell viability was 93.59\%, 98.68\%, and 103.51\% at 24, 48, and 72 h respectively. While Molloplast-B seemed nontoxic during the first three days, cell viability decreased to 88.17\% on the fourth day and slight cytotoxicity was shown. As for GC Tissue Conditioner, cell viability did not exceed 7.76\% during the entire test period. Thus, it was deemed as the soft lining material with the highest cytotoxic potency (Fig. 1).

With the soft lining materials, incubation time did not exert a significant effect on cell viability \( (p>0.05) \). However, material type strongly influenced the viability of HGFs \( (p<0.05) \). The cell viability exhibited by GC Tissue Conditioner was significantly lower than the other soft lining materials \( (p<0.05) \). Additionally, there was no interaction between incubation time and material type \( (p>0.05) \).

**Cytotoxicity assay results of hard denture lining materials**

All hard lining materials exhibited cell viability higher than 90\% throughout the entire test period. Although GC Reline Hard showed the lowest cell viability (95.05\%)
after 96 h of incubation, this value was markedly distant from any indication of cytotoxicity. Therefore, Vertex-RS, GC Reline Hard, UFI Gel Hard C, and Vertex-SC were considered as non-toxic lining materials (Table 2).

With the hard lining materials, both incubation time and material type did not significantly influence cell viability ($p>0.05$). There was also no interaction between incubation time and material type ($p>0.05$).

**DISCUSSION**

The term “cytotoxicity” is used to describe the cascade of molecular events that interfere with macromolecular synthesis, causing unequivocal cellular, functional, and structural damage. To evaluate the cytotoxicity of a material, biocompatibility testing should be performed with the most appropriate cell types to mimic the actual interaction between the material and the cells$^{[10]}$. However, the choice of cell line for *in vitro* cytotoxicity screening assays remains controversial, because the apparent cytotoxicity of a material can be significantly affected by the pre-assay preparation of the material$^{[17]}$.

Among the vast number of cell types that have been used, normal diploid human cells are the more appropriate cells for toxicity testing of materials for human use$^{[18]}$. Evidence has shown that some denture lining materials caused allergic reactions such as burning sensation in the mouth or red, swollen and painful gums$^{[19]}$. Sometimes, even oral vesicles and ulcers were formed$^{[19]}$. These materials can come in contact with the connective tissue cells when the epithelium disappears. Therefore, it is important to evaluate the cytotoxic effects of elastic denture lining materials not only on epithelial cells but also on gingival fibroblasts$^{[20]}$. For this reason, human gingival fibroblasts (HGFs) were used in the present study to evaluate the cytotoxicity of denture lining materials.

For *in vitro* cell culture models, XTT and MTT are colorimetric methods used for high-throughput drug screening and toxicity evaluation. They are based on the reduction of tetrazolium salts to colored formazans by metabolically active cells, enabling the quantitative evaluation of cell proliferation in a simple and effective manner. In this study, XTT —instead of MTT— was selected as the method for cytotoxicity testing because of these advantages of XTT over MTT: higher sensitivity, less manipulation because of elimination of solubilization step, reduced risk of error (e.g., no air bubbles from SDS or Triton X-100), and reduced incubation time$^{[13]}$.

**Soft denture lining materials**

Phthalates and other esters of aromatic carboxylic acids are used as plasticizers in acrylic soft lining materials. It was reported that esterase activity, equivalent to that in saliva, in an immersion medium for soft denture lining materials increased the rate of diffusion of plasticizers from the materials$^{[21]}$. For the plasticizer dibutyl phthalate (DBP), its average leached amount within the first day exceeded the proposed tolerable daily intake (TDI) for an average adult person by about 11–32 times$^{[22]}$. In the present study, GC Tissue Conditioner—an acrylic-based soft lining material—exhibited clear cytotoxic effect at all incubation periods. This could be because GC Tissue Conditioner contained the DBP component.

Park et al.$^{[9]}$ evaluated the cytotoxicity of short-term-use soft liners after repeated elution using the agar overlay method. Although cytotoxicity decreased after repeated elution, they recommended these materials to be used within a limited time$^{[9]}$. Further on soft lining materials, Ozdemir et al. found that a vinyl polysiloxane material exhibited heightened cytotoxic effect after 96 h of incubation$^{[23]}$. Although not previously reported for vinyl polysiloxane polymers, Mutluay and Ruyter cautioned that allergic reactions should always be kept in mind before applying fresh uncured polymer directly to the mucosa$^{[24]}$. However, contrary to these reports, El Hadary and Drummond claimed that vinyl polysiloxane materials were manifested to be non-toxic after prolonged exposure of cells to these materials$^{[25]}$.

In the present study, GC Tissue Conditioner—a temporary acrylic-based soft lining material—exhibited the highest cytotoxicity at all incubation periods. Molloplast-B, a silicone-based soft lining material, presented slight cytotoxicity at 96 h. With respect to these two materials, our findings were in accordance with El Hadary and Drummond$^{[26]}$. However, in contrast to the results of Ozdemir et al.$^{[23]}$, prolonged exposure of HGFs to Molloplast-B at 96 h in this study resulted only in slight increase in cytotoxicity—not a marked increase in cytotoxic effect as that reported by Ozdemir et al.$^{[23]}$.

A survey of published literature revealed limited information on the cytotoxic effects of soft lining materials with different compositions. In the present study, GC Tissue Conditioner was significantly more cytotoxic than Mollosil Plus ($p<0.05$), UFI Gel-SC ($p<0.05$), Molloplast-B ($p<0.05$), and Visco-gel ($p<0.05$). In light of results obtained in reported studies and the present study, temporary acrylic-based materials seemed to manifest higher cytotoxic effects than soft lining materials of other compositions. Therefore, their use should be limited to a short time period.

**Hard denture lining materials**

In the case of hard denture lining materials, information availability about their cytotoxicity is even more scarce. The polymerization of acrylic resin-based denture lining materials can be accomplished by various means, and it was numerously reported that polymerization process variables affected the cytotoxicity of these hard denture lining materials. Incomplete polymerization leads to release of residual monomer from acrylic resin-based hard denture lining materials, which in turn causes irritation, inflammation, and allergic response of the oral mucosa$^{[26]}$.

Vallittu et al.$^{[27]}$ reported that polymerization temperature and polymerization time considerably affected the residual monomer content of denture base polymers, and that auto-polymerized resins had a higher residual monomer content than heat-polymerized
resins. Similarly, Lamb et al.\textsuperscript{29} stated that residual monomer content was lower in samples polymerized at 55°C than those polymerized at 22°C. On the effect of polymerization time on cytotoxicity, Sipahi et al.\textsuperscript{29} reported that MTT assay revealed a lower viabiity of gingival fibroblasts at 48 h than at 120 h. Moreover, the autopolymerized acrylic-based material showed the lowest cell viability.

In the present study, contrasting results were obtained with autopolymerized acrylic-based hard denture lining materials tested in this study: GC Reline Hard, Ufi Gel hard C, and Vertex-SC. They consistently exhibited cell viability which exceeded 90% over the entire 96-h test period. Moreover, their cell viability results were not significantly different from that of heat-polymerized acrylic material, Vertex-RS, at all incubation periods \( p > 0.05 \). Thus, all the hard denture lining materials tested in this study exhibited high cell viability and good biocompatibility.

**CONCLUSIONS**

Within the limitations of this \textit{in vitro} study, the following conclusions were drawn:

1. GC Tissue Conditioner, an autopolymerized acrylic-based soft denture lining material, exhibited the highest cytotoxic effect at all incubation periods. It must be used with caution as it exhibited high cytotoxic potency to induce allergic reactions.

2. The remaining soft denture lining materials —namely, Mollosil Plus, Ufi Gel SC, Molloplast-B, Visco-gel, and all the hard lining materials tested in this study showed high cell viability and good biocompatibility at all incubation periods, indicating that they were safe for clinical use.

**REFERENCES**