In vitro Analysis of Cytotoxicity of Temporary Resilient Relining Materials

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ABSTRACT

Aims: The aim of this study is to evaluate the in vitro response of human gingival fibroblasts in primary cultures to two materials for temporary relining of dentures: Temporary Soft (TDV, Brazil) and Trusoft (Bosworth, USA) for 24 hours, 7 and 30 days by using a multi-parametric analysis.

Materials and methods: Each material sample (TDV, TS, Polystyrene, Latex) was prepared and incubated in a culture medium for 1, 7, and 30 days at 37°C. Human gingival fibroblasts were exposed to the extracts and cell viability was evaluated by a multi-parametric assay, which allowed sequential analysis of mitochondrial activity (XTT), membrane integrity [neutral red (NR)], and cell density [crystal violet dye exclusion (CVDE)] in the same cells. Analysis of variance (ANOVA) was used to test the interactions of the three sources of variation (material, test method, and time) with the proportions of viable cells for each relining material.

Results: Both evaluated materials (TDV and TS) had low cytotoxic effects during 1, 7, and 30 days after manipulation of the material, as assessed by all three methods used. A statistical difference was found when comparing the negative control group (latex fragments) with the other groups, which showed high toxicity and low percentage of cell viability in all tests used. There was no significant difference among other materials (p > 0.05).

Conclusion: Low cytotoxicity levels were detected by representatives of the major groups of temporary prosthetic relining materials, as evaluated by multiple cellular viability parameters in human fibroblasts.

Clinical significance: There are various soft materials on the market for relining prostheses; however, the effects of these materials on tissues need to be clarified to avoid problems for patients.

Keywords: Biocompatibility, Cytotoxicity, Dental prosthesis, Denture liners, Fibroblasts, Laboratory research.

INTRODUCTION

In removable full dentures, the appropriate transmission of masticatory forces to the support area depends on both the correct intermaxillary relationship and the adaptation of the denture base to the fibromucosa. However, even when removable dentures are properly made, some patients have difficulty in utilizing them due to the resorption of the alveolar ridge, bruxism, hyposalivation, and sensitivity. Given these aspects, resilient relining denture materials were developed to reduce the impact of masticatory forces on the mucosa, provide better adaptation of prosthetics, and greater comfort to patients.

Depending on the period of contact of the prosthesis with the support mucosa, these materials may be used in short or long term. Long-term materials are used for more than 28 days, while, for greater retention and comfort, short-term materials are used for a limited period of up to 7 days.
The temporary denture prostheses are generally supplied in the form of powder and liquid. In acrylic resin-based soft materials, the powder consists of poly(ethyl methacrylate) or related copolymers. The liquid consists of ethanol (solvent) and a plasticizer, usually an aromatic amine. After coming into contact with the oral mucosa, the prostheses become permanent; in this process, ethanol and plasticizer are lost, resulting in the degradation of the material, accompanied by changes in volume and color and hardening.4

The dissolution of ethanol mainly occurs in the first 24 hours, causing irritation to the injured tissue, especially in cases of materials with high ethanol content. It has been reported in the literature that dissolution of the plasticizer may also cause toxicity and estrogenic activity.5 6

The release of denture relining components can lead to erosion in the fibromucosa and a burning sensation in the mucosa and tongue.7 In addition, resilient denture relining materials are easily degradable, and can act as a reservoir for microorganisms, causing complications, such as problems in the pharynx and respiratory tract, especially in elderly patients with reduced immune activity.8 9

Several techniques can be used to evaluate the in vitro cytotoxicity of dental materials. Since three different parameters are evaluated in the same group of treated cells, the multi-parametric approach reported here can be used to evaluate the cytotoxicity of the materials. The XTT assay measures mitochondrial activity, the neutral red (NR) uptake assay evaluates the integrity of the cell membrane, and the crystal violet dye exclusion (CVDE) evaluates the cellular density.10 11

There are various soft materials on the market for relining prostheses; however, the effects of these materials on tissues remain unclear. The aim of this study was to evaluate the in vitro response of human gingival fibroblasts in primary cultures to two materials for temporary relining of dentures: Temporary Soft (TDV, Pomerode, Santa Catarina, Brazil) and Trusoft (Bosworth, Skokie, Illinois, USA); a standardized multi-parametric assay with three sequential tests of cell viability: XTT, NR and CVDE, over a period of 1, 7, and 30 days was used.

**MATERIALS AND METHODS**

This study was approved by the Ethics Committee of Antônio Pedro University Hospital/Fluminense Federal University (1.188.109).

The following samples were utilized in this study: Temporary Soft (TDV, Pomerode, Santa Catarina, Brazil), Trusoft (Bosworth, Skokie, Illinois, USA), dense polystyrene beads (P) as positive control, and latex fragments (L) as negative control. The materials (Temporary Soft and Trusoft) were prepared as described in the manufacturers’ instructions (Table 1). The samples in the shape of discs (10 × 1 mm) were prepared from previously sterilized metal matrices as described for irregularly shaped solid devices in ISO 10993-5:2014.2 0.1 g of each material was immersed in 1 mL of Alpha-MEM culture medium without FCS (Gibco, Cergy-Pontoise, France) and incubated for 24 hours at 37°C in a humidified chamber. Three experimental groups were obtained by examining the samples immediately, 7 and 30 days after preparation.

**Multi-parametric in vitro Assay**

The extracts were prepared according to ISO 10993-12,3 and cytotoxicity of the prepared samples was assessed in vitro with a multi-parametric assay kit (Cytotox-In, Xenometrix, Allschwil, Germany), which evaluates three different cell viability parameters sequentially in the same cell culture.11

Primary cultures of human fibroblasts were obtained from the collection of the Laboratory of Experimental Cell Culture (LECcel) of the Federal Fluminense University (UFF). The cells were cultured in a D-MEM medium (Cultilab, Campinas, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS) and two antibiotics (Penicillin 10,000 U/mL and Streptomycin 10 mg/mL). The human fibroblasts with an initial density of 10,000 cells per well were sub-cultured for 24 hours at 37°C in 96-well culture plates, and subsequently exposed to a conditioned medium for 24 hours. The negative control group was exposed only to the culture medium (D-MEM). Each

### Table 1: Materials tested

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Composition</th>
<th>Lot</th>
<th>Preparation mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporary soft</td>
<td>TDV</td>
<td>Powder: EMA, benzoyl peroxide, starch, and organic pigments. Liquid: Denatured alcohol, DBP, and mint essence.</td>
<td>0419/1113</td>
<td>Powder: 2 cm³ (mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liquid: 1 cm³ (mL)</td>
</tr>
<tr>
<td>Trusoft</td>
<td>Bosworth</td>
<td>Powder: Pigmented polyethylene methacrylate, cadmium pigments (pink pigment) Liquid: Ethyl alcohol, plasticizer</td>
<td>1211-495</td>
<td>Powder: 1.06 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liquid: 1 mL</td>
</tr>
</tbody>
</table>
condition was tested in three replicates and three different assays.

After observing sub-confluence at 24 hours, the culture medium was removed and replaced by 180 μL extract of either material (Temporary Soft, Trusoft, Polystyrene, or Latex). Fetal bovine serum (10%) was then added to each well, thereby obtaining five replicates of each experimental condition.

After the cells had been exposed to the extracts for 24 hours, the CytoTox® kit – XTT-NR-CVDE (KXRCV 96,300/310, Xenometrix Inc., Allschwil, Switzerland) was used to assess cytotoxicity.

**Mitochondrial Activity**

Viable cells require an intact respiratory chain and mitochondrial membrane. The multi-parametric assessment begins with XTT (2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxy aniline salt), a tetrazolium salt, i.e., converted to formazan through a succinate dehydrogenase system in the mitochondrial respiratory chain of the viable cells. The conversion of water-soluble yellow tetrazolium XTT salt into orange formazan was monitored by measuring the absorbance at 480 nm on a UV/Vis microplate reader (Synergy II, BioTek Instruments, Winooski, Vermont, USA).

**Membrane Integrity**

The cells subjected to the XTT assay were washed and used in the NR uptake assay, which determines the viable cell count through cellular membrane integrity. The NR test is a survival/cell viability assay based on the ability of the cell to incorporate and retain NR through its lysosomes, which accumulate in its internal membrane. The cells were fixed after 3 hours of exposure to the dye. Neutral red was extracted and measured by optical density (OD) of the supernatant at 540 nm, which is directly related to the proportion of viable cells.

**Cellular Density**

After NR assay, the fixed cells were washed and evaluated via the CVDE, which quantifies nuclear DNA to assess cell density. After washing/removal of the excess dye, the absorbance at 540 nm is directly proportional to the number of adherent cells in each well.

**Statistical Analysis**

The analysis of variance (ANOVA) was used to test the interactions between three sources of variation (material, assay method, and time) with the proportions of viable cells for each relining material, when compared to the control group. Statistical decisions were made at an alpha = 0.05 significance level. The Tukey’s test was performed to compare all groups at the same time. All statistical analyses were performed with the software SPSS 10.0 [Statistical Package for the Social Sciences (SPSS), Inc., Chicago, Illinois, USA].
RESULTS

Graphs 1A to C shows the cell viability, as compared with dense beads of polystyrene (positive control) and latex fragments (negative control), measured by the three tests (XTT, NR, CVDE) after 1, 7, and 30 days of exposure to the tested resilient relining materials.

As it can be seen in Graphs 1A to C, in all the three tests, both materials TDV and TS had low cytotoxic effects with > 20% survival after 1, 7, and 30 days of exposure to the materials and were in relation to the negative control group. Statistically significant reduction in the cell viability of the negative control group (latex fragments) when compared with the other groups, in all the three tests, suggests high toxicity. There was no significant difference among the other materials (p > 0.05).

When the membrane integrity was assessed by XTT assay at different time periods, cellular viability after exposure to TS was greater than the negative control group, whereas cellular viability after exposure to TDV revealed a significant difference between the groups (p < 0.05).

DISCUSSION

Absorption of certain substances released in high concentrations by odontological materials poses health hazards to the patients; therefore, assessment of biological and toxicological safety of odontological materials should be a prerequisite for clinical use. In vitro cytotoxicity tests are simple, reproducible, effective, and suitable for use in the assessment of the biological properties of odontological materials.12,13

Choice of the cell type for the in vitro cytotoxicity tests remains controversial and a variety of cells have been used in various studies.14 Although the use of primary cell lines presents disadvantages, such as the requirement of having access to a laboratory facility at the time of tissue collection, time-consuming cell isolation, a generally low number and limited lifespan of cells,15 this methodology was chosen due to its ability to simulate a clinical situation closer to reality. Moreover, it is important to evaluate the cytotoxic effects not only on epithelial cells but also on gingival fibroblasts.16

In the present study, the cytotoxicity of two commercially available temporary relining materials was tested; while TDV has been recently launched in the market and poorly studied, TS is already used worldwide. The in vitro methodological approach employed here differs from most previous works on these materials in the implementation of the multi-parametric assay with three different cell viability tests and the use of a primary culture of human cells.11 Three different parameters could be evaluated for the same group of treated cells. This is relevant because the results obtained with only one method may be often over- or under-estimated, due to interference or methodological limitations.10,11,17,18

The silicone-based relining materials (TDV and TS) evaluated in the present study did not show significant cytotoxicity when in contact with human gingival fibroblasts. This finding is in agreement with Atay et al.19 who analyzed nine soft and hard relining materials against human gingival fibroblasts, and observed that the soft materials were not cytotoxic, presented high cell viability, and good biocompatibility. Further, in agreement with the reports of Tay et al20 on different resilient denture materials (Dentusoft, Dentuflex, Trusoft, Ufi-Gel P, and Lucitone-550), Trusoft material as slightly cytotoxic.

On comparing the cytotoxic effect of silicone- and acrylic-based relining materials, it can be concluded that there is a greater possibility of cytotoxic effect when the acrylic-based soft denture relining materials are used.19,21 These materials also present higher water absorption and solubility in addition to promoting lower tensile strength. Therefore, silicone-based materials have higher rates of clinical success.22

In a study comparing cytotoxicity of silicone-based relining materials (Coe-Comfort; Coe-Soft; Visco-gel; and Sofreliner) using the XTT assay in mice fibroblasts for 24, 48, and 72 hours, it was concluded that depending on their composition, time alters resistance and cytotoxicity of soft materials for dentures.23 This has also been demonstrated by Inoue et al,24 who, after testing experimental soft relining materials, found that composition affects their solubility, viscosity, and water absorption.

A study evaluating the resistance of denture relining materials up to 12 months concluded that all materials tested had an acceptable adhesion to denture base and could be suitable for long-term use.25 Ozdemir et al26 studied cytotoxicity of rigid and resilient denture materials (Visgo-Gel, Ufi Gel P, Sofreliner, Coe-Soft, and Molloblast-B) for periods of 24, 72, and 96 hours, and found that the Sofreliner material showed cellular cytotoxicity after 96 hours. This observation indicated the necessity to analyze the materials for longer periods, and prompted this study, where time periods of 7 and 30 days were used.

The type of polymerization of the relining material may be related to its cytotoxic effects. A systematic review of the literature27 suggested that thermo-polymerized resins present reduced cytotoxicity when compared to auto-polymerizable, photo-polymerizable, or dual materials. This fact was also confirmed by Sheridan
et al., who, when evaluating chemically and thermooxidative relining materials on human gingival fibroblasts, found reduced cytotoxicity of thermo-polymerized materials.

As majority of the studies concerning cell viability of relining materials can come from the chosen experimental model, it would be useful if researchers chose models that use primary human cells and multi-parametric evaluations.

CONCLUSION

It can be concluded that representatives of the major groups of temporary prosthetic relining materials present low levels of cytotoxicity when evaluated by three different cell viability parameters on human fibroblasts.

CLINICAL SIGNIFICANCE

There are various soft materials on the market for relining prostheses; however, the effects of these materials on tissues need to be clarified to avoid problems for patients. The cytotoxicity of two commercially available temporary relining materials was tested in this study, and it was concluded that both can be used with safety because they have low cytotoxic effects.

ACKNOWLEDGMENT

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REFERENCES


