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EVALUATION OF BIOLOGICAL RESPONSES OF DENTURE SOFT LINERS ON L929 FIBROBLAST CELL LINES- AN EX-VIVO STUDY

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Abstract:

Aim: The aim of the study was to evaluate the biocompatibility of denture soft liners using a fibroblast cell line.

Materials and methods: The effects of two denture liners (acrylic-based GC Soft Liner and silicone-based GC Reline Soft) on L929 fibroblast cell lines were investigated. Eluates from the material specimens were applied directly on the cells and cytotoxicity of specimen eluates and cell viability were evaluated by MTT assay and changes in cell morphology were evaluated by direct contact assay and inverted phase contrast microscopy. Controls were cells in culture medium without eluates or specimens.

Results: GC Soft Liner(acrylic-based soft liner) showed lower cell viability and more cytotoxicity than GC Reline Soft (silicone-based soft liner).

Conclusions: Silicone based denture soft liners are comparatively non cytotoxic to fibroblasts.

Introduction

The denture soft liners act as a cushion between the denture base and residual ridge. They are often used to provide better fit and comfort for patients who cannot tolerate conventional hard denture bases because of excessive residual ridge resorption, bruxism, xerostomia, and fragile supporting mucosa[1]. They have been developed to help patients when their oral mucosa is damaged or affected due to ill-fitting dentures or post-implant surgery[2]. These soft liners may release components as residual monomers, plasticizers, degradation products[3,4] and alcohol[5,6]. Although reports have indicated that these materials leach monomers and other components that do affect their biocompatibility, there is a little information on what cell molecules may be implicated in these materials. Only a few studies evaluated the effects of direct contact between cells and soft liners[7–10]. Therefore, the aim of this study was to evaluate the biocompatibility of denture soft liners using a fibroblast cell line.

Materials and Methods

Materials

The materials selected for this study, types, manufacturers, powder/liquid ratio, compositions

and polymerization/gelation time are presented in Table 1.

Sample preparation

The specimens (discs of diameter 10 mm × thickness 1 mm) of each material were prepared under aseptic conditions. The materials were mixed according to the manufacturers' instructions and inserted into metal molds; pressure was applied until the reaction was complete. Samples were exposed in UV irradiation for 30 minutes and were directly taken for the analysis.

Cell Culture

For biological evaluation, L929 (Mouse Fibroblast) cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in DMEM supplemented with 10% FBS and containing the antibiotics penicillin, streptomycin and amphotericin B (5000 units) in a humidified incubator at 5% CO2 at 37 ± 0.20C. The cells were regularly monitored by phase contrast inverted light microscopy. The medium was changed once in three days. The confluent monolayer was sub-cultured and maintained for further studies[11]

Evaluation of the toxicity of specimen eluates by MTT assay

The cytotoxicity of the specimens was evaluated as per ISO10993-5 on L929 (Mouse Fibroblast) cell culture. The cells were seeded onto a 48 well plate and incubated. After attaining confluency, the sterile specimens were added to the cell seeded plate. Culture medium without test specimens was also incubated under the same conditions and served as control. The percentage of the surviving fibroblast cells were quantified by the MTT assay and the morphological changes of the cells were monitored by phase contrast microscopy[12].

MTT assay is carried out to measure mitochondrial cellular metabolism (viability) and number of viable cells. MTT assay is based on the capability of metabolically active fibroblast cells to reduce the yellow water-soluble tetrazolium salt (MTT) to

Table 1: Materials evaluated in this study.

Product	Type	Manufacturer	Powder/ liquid ratio	Composition (manufacturer supplied)	Polymerization/ gelation time
GC SOFT LINER	Auto-polymerizing acrylic-based soft liner	GC, Japan	2.2 g/ 1.8 g	Powder : Poly ethyl methacrylate - 100% Butyl phthalyl butyl glycolate - 8.9% Dibutyl phthalate - 4.3% Liquid : Ethyl alcohol - 14.8%	5 min
GC RELINE SOFT	Auto-polymerizing silicone-based soft liner	GC, America	Auto dispensing system (1:1)	Catalyst paste : Vinyl-terminated polydimethylsiloxanes Platinum catalyst. Base paste : Vinyl-terminated polydimethylsiloxanes Hydride-terminated polydimethylsiloxanes	7 min

purple formazan crystals using the mitochondrial enzyme succinate dehydrogenase (SDH). The intensity of purple colour so formed is proportional to the number of viable cells.

Following the experiment, the culture was washed with 1 x PBS and then 200 μl MTT solution per ml culture (MTT 5 mg/ml dissolved in PBS and filtered through a 0.2 μm filter before use) were added. The whole content was again incubated at 37oC for 3h and 300 μl DMSO were added to each culture well. The whole content was incubated at room temperature for 30 min until all cells were lysed and a homogenous colour was obtained. The solution was centrifuged for 2 min to sediment cell debris. The optical density (OD) was measured spectro-photometrically at 540 nm. Cells treated with MTT solution without the sample was used as control. The % viability was calculated as follows:

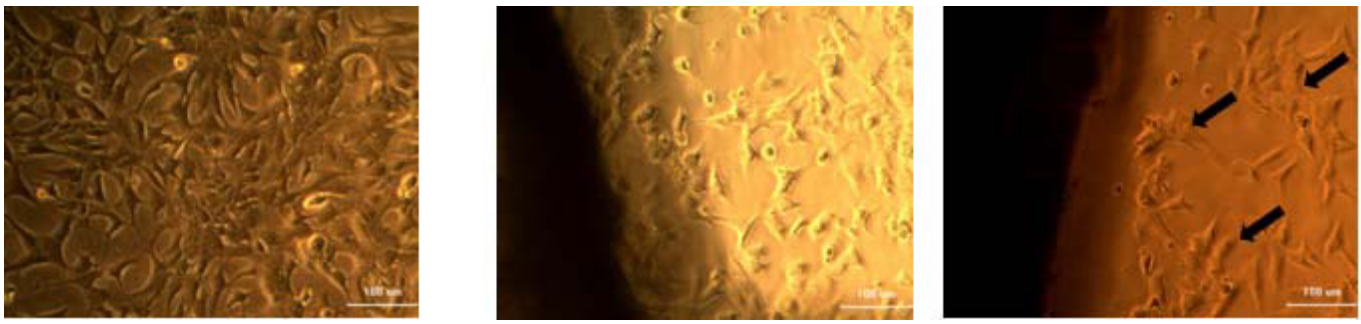
% Viability = (OD of test)/ (OD of control) X 100

Direct Contact Method

The cytotoxicity of specimens under the direct contact of cell was determined by direct contact assay. L929 (Mouse Fibroblast) cells (1x104 cells/m) were seeded on to a 48 well plate and allowed to proliferate for 24 h to form a sub-confluent layer. Then the specimens were placed over the monolayer and allowed to proliferate in a CO_2 incubator. After 24 hours of incubation, cells were evaluated for changes in morphology with respect to a control (cells grown without specimens) under inverted phase contrast microscope (Olympus CKX41) attached with an imaging camera. The images were captured using imaging software Optika vision-pro[13-15].

Table 2: Mean optical density value and% cell viability after cells exposure to 24 hour eluates from specimens.

SAMPLES	OD Value I	OD Value II	OD Value III	Mean OD Value (540 nm)	% viability
CONTROL	0.9801	0.9816	0.9810	0.9809	100
GC RELINE SOFT					
(Silicone-based)	0.7477	0.7144	0.7480	0.7367	75.10
GC SOFT LINER					
(Acrylic-based)	0.5990	0.5990	0.5845	0.5942	60.57



(a) (b) (c)

Figure 1: Panel of inverted phase contrast microscopic images of L929 fibroblast cells after 24 hrs of direct contact for (a) Control (cells grown without specimens), (b) GC Reline Soft (silicone-based soft liner) and (c) GC Soft Liner (acrylic-based soft liner)

Statistical Analysis

All statistical analyses were performed with SPSS for Windows (release 17.0, SPSS Inc., Chicago, IL, USA). Data from MTT tests (with eluates and direct contact) were analyzed by two-way ANOVA followed by Tukey's test, with $P \leq 0.05$.

Results

Mean optical density value and percentages of cell viability relative to controls, obtained in the MTT assay, are shown in Table 2. For L929 fibroblast cells, exposure to the 24 hour eluates from GC Soft Liner (acrylic-based soft liner) resulted in significantly lower percentages of cell viability than those obtained with the 24 hour eluates from GC Reline Soft (silicone-based soft liner).

Figure 1 show the inverted phase contrast microscopic images of L929 fibroblast cells for controls (cells grown without specimens) and experimental conditions (cells grown in direct contact with the soft liner specimens). As shown in Figures 1(a), control cells displayed their characteristic spindle-shaped morphology and undergoing mitosis. GC Reline Soft (silicone-based soft liner) resulted in a significantly less number of necrotic cells after 24 hrs of direct contact with the L929 fibroblast cells [Figure 1(b)] when compared to GC Soft Liner (acrylic-based soft liner) [Figure 1(c)] but more when compared to control.

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[16]. L929 fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests[17]. The L929 fibroblast cell lines used in this study are sensitive to dental monomers and plasticizers that can be released from polymer materials.

The results from MTT assay showed that the GC Soft Liner (acrylic-based soft liner) was more cytotoxic to L929 fibroblast cells compared to GC Reline Soft (silicone-based soft liner). The absence of significant reductions in cell viability after exposure to eluates from the materials does not exclude the possibility of damage to delicate cell structures. Thus, in the present study, microscopic analysis of cellular morphology was performed. Inverted phase contrast microscopic images were used to assess changes in cell morphology induced by direct contact of L929 fibroblast cells with the soft liner specimens. For direct contact (24 hr time period), the silicone-based soft liner GC Reline Soft was less cytotoxic to the L929 fibroblast cells, while the acrylic-based soft liner GC Soft Liner exerted greater effects.

Silicone-based soft liners are similar to polyvinylsiloxane-based impression materials[1] and polymerize by an addition reaction with no by-products, such as alcohol. It has been found that, although components such as monomers and phthalic plasticizers were released by soft liners, the silicone-based materials were generally more stable, releasing smaller quantities than the acrylic-based softliners[3,4]. Thus, it can be supposed that GC Reline Soft exerted less-pronounced cytotoxic effects on the L929 fibroblast cell lines due to a lower release of residual components.

The materials evaluated also contain plasticizers such as dibutyl phthalate (DBP) in GC Soft Liner (acrylic-based soft liner). The toxicity of phthalates is caused by their metabolite methoxyacetic acid (MAA), through mechanisms that involve ROS generation and DNA and mitochondrial damage[18]. The exposure of L929 fibroblast cells to DBP for 24 h[19] led to a reduction in DNA synthesis, cell metabolism, and viability. Thus, it is likely that the cytotoxic effects observed here for GC Soft Liner (acrylic-based soft liner) are related, at least in part, to the presence of plasticizers in its composition.

Other studies also found that the direct contact between different cells and denture liners and tissue conditioners resulted in cytotoxic effects,

such as zones of growth inhibition, cell lysis[7], and reduced cell viability[20]. Kruni'c et al. observed lower cytotoxicity for the silicone-based soft liners, compared with the acrylic-based materials[21]. Park et al. 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[22]. Ozdemir et al.[16] found that a vinyl polysiloxane material exhibited heightened cytotoxic effect after 96 h of incubation. Munksgaard[23] showed that the leaching of phthalate during the first day of use exceed tolerable daily intake by 11–32 times for different materials and this may cause undesirable biological effects. In an another study, Munksgaard[24] reported that an esterase activity, equivalent to that in saliva in the immersion medium for soft lining materials increased the rate of diffusion of plasticizer from the materials. Mutluay and Ruyter[25] pointed that, although not reported previously for vinyl polysiloxanes, allergic reactions should be always kept in mind because of applying fresh uncured polymer directly to the mucosa. Dahl et al.,[26] investigated the in vitro cytotoxicity of denture relining materials using cell culture tests and a test for irritation mechanisms. They stated that five of the tested materials were slightly or moderately cytotoxic in the contact test. They also reported that nine of the eleven products showed cytotoxic response in the MTT test using extracts of the test specimens.

Although the results obtained from in vitro studies cannot be directly extrapolated to clinical situations, it is important to consider whether the changes observed in the present investigation are cumulative and become increasingly pronounced with time. This could make the cells more susceptible to subsequent challenges, such as direct contact with newly applied soft liner materials. It is important to note that, due to a progressive loss of plasticizers and alcohol, the soft liners need to be replaced at regular intervals. Although these replacements will prevent trauma and colonization of the material by microorganisms, they are performed directly in the mouth, increasing the exposure of tissues to the leached components, which, even in low concentrations, can lead to chronic adverse effects on the oral mucosa. Even if not causing acute cytotoxicity, the continuous release of such substances may compromise tissue homeostasis and repair, which are particularly important given that some softliner materials are applied over inflamed supporting tissues and during the healing phase in immediate dentures or dental implants[27].

Conclusions

Within the limitations of this study, the following conclusions were drawn:

- The 24 hour eluates from GC Soft Liner (acrylic-based soft liner) were more cytotoxic to L929 fibroblast cell line tested compared to GC Reline Soft (silicone-based soft liner).
- The silicone-based material, GC Reline Soft, caused less reduction in cell viability when in direct contact with the L929 cell lines tested, for 24 hours.

All these findings are importance because the soft liners are often applied on areas of ulcerated tissue or after surgery, where both macrophages and fibroblasts play important roles in the healing process[27].

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