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Cytotoxicity of four denture adhesives on human gingival fibroblast cells

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Abstract

Objective. The purpose of this study was to compare the cytotoxicity of four denture adhesives on human gingival fibroblast cells. ***Materials and methods.*** Immortalized human gingival fibroblasts were cultured with one of four different denture adhesives, Polident, Protefix, Staydent or Denfix-A, which was placed in insert dishes (10% w/v concentration) for 48 h. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and flow cytometric apoptosis assay were used to evaluate cell viability and apoptosis rates. The fibroblasts were also examined under a scanning electron microscope. ***Results.*** The MTT assay showed that all denture adhesives resulted in a significantly lower cell viability compared to the control cells propagated in normal culture medium ($p < 0.05$), with Staydent demonstrating the lowest cell viability. According to the flow cytometric apoptosis assay, Staydent and Protefix showed significantly higher apoptosis rates than the control group ($p < 0.05$), whereas Polident and Denfix-A did not demonstrate any significant differences ($p > 0.05$). Staydent showed the highest apoptosis rate. Scanning electron microscopy showed that the cells of the Staydent group underwent cytoplasmic membrane shrinkage, with cell free areas containing residual fragments of the membrane of dead cells. ***Conclusions.*** The four denture adhesives evaluated in this study imparted cytotoxic effects on human gingival fibroblast cells. Staydent showed the highest toxicity.

Key Words: *cytotoxicity, denture adhesive, flow cytometric apoptosis assay, human gingival fibroblast, MTT assay, scanning electron microscope*

Introduction

Denture adhesives are materials used to attach a denture to the oral mucosa and improve its retention and stability [1]. Although numerous dentists discourage the use of these materials, even viewing it as a poor reflection of their clinical skills, the use of denture adhesives have continued to spread [2]. Early studies have shown that the adhesives allowed the prolonged use of ill-fitting dentures and promoted bone resorption, although more recent studies indicate that, when used properly, denture adhesives may enhance clinical outcomes [3,4].

Denture adhesives increase the retention of dentures by filling up the space between the denture and the oral mucosa by swelling and increasing the coefficient of surface tension of the fluid film between the denture and the oral mucosa [5]. Even in a properly fitted denture, denture adhesives increase denture stability and reduce tissue irritation and frequency of adjustments [6,7]. In ill-fitting dentures, adhesives reduce mucosal discomfort, sore spots and gum inflammation [8,9].

Among the various properties of denture adhesives, the most important is that of biocompatibility with the soft tissues of the mouth. Components leached out from the denture adhesives might be ingested by the denture user or cause irritation to the oral mucosa [1]. However, studies on denture adhesives have mainly focused on its functional effectiveness and there have been relatively fewer studies on their cytotoxicity or irritation potential [10]. Therefore, the purpose of this

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study was to compare the cytotoxicity of four denture adhesives on human gingival fibroblast cells.

Materials and methods

Denture adhesives

Four commercially available denture adhesives tested in this study are listed in Table I.

Cell culture

Human gingival fibroblast-1, HGF-1 (ATCC CRL-2014) cells were maintained in DMEM medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (Gibco), 100 U/mL penicillin, 100 mg/mL streptomycin (Pen Strep, Gibco) and 10 mM HEPES (*N*-2-hydroxyethylpiperazine, *N*-2-ethanesulfonic acid) (Gibco). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay

The effect of denture adhesives on the viability of HGF-1 cells was examined. The cells were seeded at a density of 1×10^5 cells/well in 24-well plates. After an incubation of 24 h, the medium was removed and 2 mL of fresh culture medium was added to each well. Insert dishes were placed in the 24-well plates. In the insert dishes, denture adhesives were placed so that the diluted concentrations (w/v) of the denture adhesives to the culture medium were 10%. The plates were incubated for 48 h. Afterwards, 5 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent (1 mg/mL, Sigma Chemical, St. Louis, MO) diluted in a 1:20 ratio with the culture media was added to all wells and incubated for 3 h. Afterwards, 2 mL of dimethyl sulfoxide (DMSO, JUNSEI Chemical Co., Tokyo, Japan) was added to each well and the plate was left with a cover in the dark for 20 min at room temperature. A spectrophotometer (PowerWave x340, Bio-Tek instruments, Winooski, VT) was used to read the optical density of the formazan precipitate at a wavelength of 595 nm.

Flow cytometric apoptosis assay

Cells were grown in 6-well culture plates at a density of 4×10^5 cells/well. After an incubation of 24 h, the medium was removed and 2 mL of fresh culture medium was added to each well. Insert dishes were placed in the 6-well plates. In the insert dishes, denture adhesives were placed so that the diluted concentrations (w/v) of the denture adhesives to the culture medium were 10%. The plates were incubated for another 48 h.

Cells were collected by trypsinization (0.05% trypsin-EDTA, Gibco, Grand Island, NY) and washed once with ice-cold phosphate-buffered saline (PBS, pH 7.4; Gibco, Grand Island, NY). Afterwards, the cells were treated with Annexin V-FITC and PI KIT (Invitrogen, Eugene, OR). The samples were analyzed on a fluorescence-activated cell sorter (FACS) flow cytometer (Beckton Dickinson, San Jose, CA).

Analysis of cell morphology by scanning electron microscopy (SEM)

The cells were seeded at a density of 4×10^5 cells/well in 6-well plates. After an incubation of 24 h, the medium was removed and fresh culture medium was added to each well. Insert dishes were placed in the 6-well plates. In the insert dishes, denture adhesives were placed so that the diluted concentrations (w/v) of the denture adhesives to the culture medium were 10%. The plates were incubated for another 48 h. At the end of the experimental period, the wells were removed and prepared for SEM. The

Table I. Manufacturers, lot numbers and composition of the denture adhesives tested in this study.

Denture adhesive	Manufacturer	Lot number	Composition
Polident® Denture Adhesive Cream	Stafford Miller, Dungarvan, Ireland	V11112A	Ca and Na salts of the copolymer of PVM-MA, poly (Methylvinylether/Maleic Acid) sodium-calcium mixed partial salt, carboxymethylcellulose, petrolatum, mineral oil, spray dried spearmint, spray dried peppermint, propyl hydroxybenzoate, erythrosine C145430
Protefix® Adhesive Cream	Queisser Pharma, Flensburg, Germany	007011	Ca and Na salts of the copolymer PVM-MA and calcium salts of the copolymer of methyl vinyl ether and maleic acid anhydride, carboxymethyl cellulose, paraffin, Vaseline, silica, menthol, azorubin, p-hydroxy-benzoic acid methyl ester
Staydent Adhesive Cream	Sheffield, New London, CT	00715	Petrolatum, Cellulose Gum, Calcium/Sodium PVM/MA Copolymer, Mineral Oil, Propylene Glycol, Diazolidinyl Urea, Methylparaben, Propylparaben, Red 27 Lake
Denfix-A™ Denture Adhesive Cream	Helago-Pharma GmbH, Bonn, Germany	012391	Cellulose gum, paraffinum liquidum, calcium/sodium PVM/MA copolymer, petrolatum, aroma(chamomile)

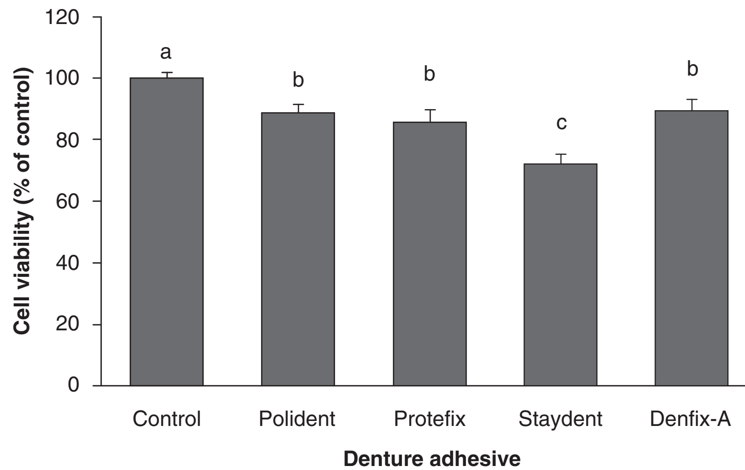

Figure 1. Cell viability of HGF-1 cells cultured with denture adhesives in insert dishes (10% w/v concentration) for 48 h was compared to that of the control (culture medium) using the MTT assay. *Different superscripts denote statistical significance ($p < 0.05$).

cells were pre-fixed overnight by 2.5% GTA + 2% PFA in PBS (pH 7.2) at 4°C and were washed with the PBS for 10 min three times. The cells were post-fixed with 1% osmium tetroxide for 90 min and washed with tertiary distilled water for 10 min three times. The cells were dehydrated in a step-wise fashion by serial incubation in increasing concentrations of ethanol: 70% (15 min), 80% (15 min), 90% (15 min), 95% (15 min), 100% (15 min), 100% (15 min) and 100% (20 min). The specimens were air-dried overnight and sputter coated with gold palladium by means of an ion sputtering coater at 20 mA for 30 s twice and observed under an S-4700 FESEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 15.0 kV and a working distance of 12 mm. A representative image of the specimens from each group at 200× magnification was used for the analysis.

Statistical analysis

For all experiments, triplicates were set up for each condition. Statistical differences between control and the cultures exposed to the adhesives were analyzed by ANOVA using the SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL). Duncan analysis was used for *post-hoc* analysis; $p < 0.05$ was considered significant.

Results

MTT

Results of the MTT assay are shown in Figure 1. All denture adhesives showed significantly lower cell viability compared to that of the cells cultured in normal culture medium ($p < 0.05$). The Staydent denture adhesive showed the lowest cell viability.

Flow cytometric apoptosis assay

The results of the apoptosis assay by flow cytometry are shown in Figure 2. Staydent and Protefix showed significantly higher apoptosis rates than the control group ($p < 0.05$), whereas Polident and Denfix-A did

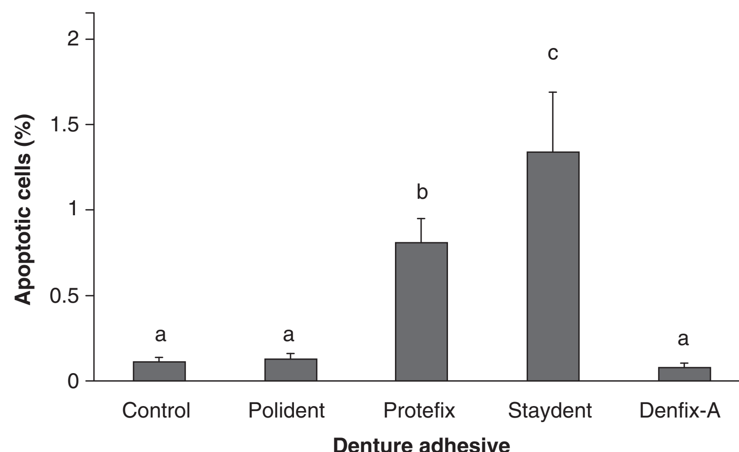

Figure 2. The percentage of HGF-1 cells in apoptosis when cultured with adhesives in insert dishes (10% w/v concentration) for 48 h. *Different superscripts denote statistical significance ($p < 0.05$).

not demonstrate significant differences compared to the control group ($p > 0.05$). Staydent showed the highest apoptosis rate.

SEM

The SEM images of the cultured cells are shown in Figure 3. In the control group, cells exhibited large cytoplasmic extensions, whereas in the Staydent group, fewer fibroblasts remained adhered to the plate surface. Cell membrane shrinkage was observed and cell-free areas with residual fragments of membrane from dead cells were observed. All the other denture adhesives also showed relatively lower cell densities compared to the control group.

Discussion

The cytotoxicity of four different commercially available denture adhesives was investigated using human gingival fibroblasts, which are the cells that come into contact with denture adhesives in clinical situations. In this study, immortalized human gingival fibroblasts, which can continuously divide without the onset of senescence, were used because of the following difficulties in the use of cell lines obtained from primary fibroblast cultures: donor availability, ethical approval issues, time consuming procedures and senescence after several passages in serial sub-culture [11]. All denture adhesives included in the study demonstrated significant cytotoxicity, with Staydent showing the highest level of cytotoxicity.

The MTT assay is a well-established method of determining cell viability and it has been used in previous studies to test the cytotoxicity of denture adhesives because it is simple, fast and objective [2,8,12]. In the present study, the four denture adhesives showed a significantly lower number of viable cells compared to that of the control. On the other hand, unlike the MTT assay, the apoptosis assay by flow cytometry detects the cells that have entered the apoptosis pathway [13], which eventually result in cell death [14]. In both assays, Staydent showed the lowest cell viability and the highest apoptosis rate, indicating that this specific denture adhesive demonstrated the highest level of cytotoxicity, inducing both necrosis and apoptosis in exposed cells.

It has been suggested that the components leaching out of denture adhesives are cytotoxic to tissues of the mouth [3] and DeVengencie et al. [12] reported that the bactericidal preservatives in denture adhesives may be the source as they may also be toxic to

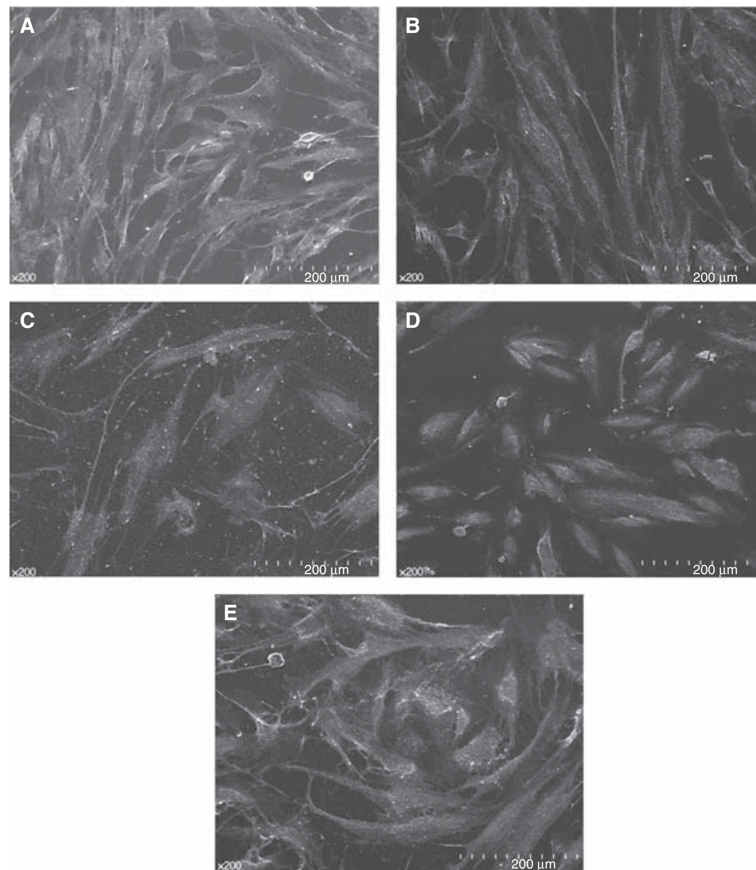

Figure 3. SEM images of HGF-1 cells cultured with adhesive adhesives in insert dishes (10% w/v concentration) for 48 h. (A) Control, (B) Polident, (C) Protefix, (D) Staydent, (E) Denfix-A. Large cytoplasmic extensions are seen in the control (A), while cytoplasmic membrane shrinkage is observed in Staydent (D).

fibroblasts. Ekstrand et al. [5] linked formaldehyde, which has been used as a preservative, to cytotoxicity. Some studies also suggested the lack of formaldehyde component as a possible explanation of the reduced cytotoxicity of the denture adhesives tested [2,12]. The unique components of Staydent include diazolidinyl urea, propylparaben and methylparaben, which are all preservatives. Previous reports have shown that parabens are relatively non-toxic [15,16]. On the one hand, diazolidinyl urea is a formaldehyde releaser, which has been reported to impart cytotoxic effects [17]. In addition, formaldehyde has been reported to cause apoptosis in some cells [18]. It may be inferred that diazolidinyl urea is the cause of the significantly lower cell viability and increased apoptosis observed in Staydent.

Another interesting result in this study is the fact that, while Protefix showed a significantly higher apoptosis effect compared to Polident and Dentfix-A, their cell viability were not significantly different among one another. It is difficult ascertain the cause of increased apoptosis effect of Protefix, but one possible cause is its silica ingredient. The effects of silica nanoparticles have been not been directly tested on human gingival fibroblasts, to the best of the authors' knowledge, but they have been reported to induce apoptosis in other human cells [19].

The current findings are not in complete agreement with the results of previous studies [3,10]. In a study by de Gomes et al. [3], the cytotoxicity of Polident and Protefix was evaluated and only Polident showed a significantly lower cell viability when mouse fibroblasts were in direct contact or in indirect contact with the denture adhesives, whereas Protefix did not [3]. Al et al. [10] also reported that Protefix cream was not cytotoxic to mouse fibroblasts according to MTT assay [10]. This is in disagreement with the current findings, as Protefix showed significantly lower cell viability. This is probably due to the higher concentrations and longer time durations used in the current study. The level of cytotoxicity is dependent on the exposure times and concentrations [3,12] and time is needed too for the constituents to be leached out of the denture adhesives [3].

Previous studies have shown that, when fibroblasts are exposed to cytotoxic dental materials, there was a decrease in cytoplasmic volume associated with shrinkage of the actin cytoskeleton and a reduction of adherent fibroblasts could be observed [3,20]. Similar findings were evident in the present study, as HGF-1 cells exhibited a large cytoplasm while exposed to the dental material, whereas, in the Staydent group, fewer fibroblasts remained adherent to the plate surface, with obvious cytoplasmic shrinkage. This is consistent with previous studies, as Staydent showed the highest level of cytotoxicity in both the MTT and flow cytometric apoptosis assays.

Unlike liquids, cream-type denture adhesives cannot be easily mixed with the culture medium. In previous studies, the cells were cultured directly on cream-type denture adhesives in direct cytotoxicity assays and, in indirect cytotoxicity assays, the extracts of the denture adhesives were obtained by incubating the culture medium and the denture adhesive together before adding the cell culture according to international standards [2,3,10]. The use of insert dishes allowed the denture adhesives to remain suspended in the culture medium, while not in direct contact with the HGF-1 cells on the plate surface. Extracts from the denture adhesives were homogeneously mixed with the culture medium to immediately expose the human gingival fibroblasts without a separate extraction step.

Exposure time and the concentration of denture adhesives are also important in the experimental design as they both affect the level of cytotoxicity. In the present study, 10% concentration (w/v) and a 48-h exposure were used, which may be a relatively high concentration and long exposure time compared to some studies that used 1% concentration and 24-h exposure time [8,10,12]. However, considering that only a thin layer of saliva coats the gingiva underneath the denture adhesive, higher concentrations might be necessary to fully represent the actual *in vivo* conditions. With the use of insert dishes, the slowly released extracts are immediately exposed to the HGF-1 cells rather than being accumulated before exposure. In addition, higher concentrations are usually used for cream-type adhesives compared to powder type adhesives [3]. Moreover, while the ideal denture adhesives should be retained in the mouth for 12–16 h [2], patients often use these for an extended period of time, thus the 48-h exposure time was chosen for this study. A previous study involving the indirect contact test using a cream form of a denture adhesive used the same concentration and exposure time employed in the present study [2]. However, one of the limitations of this study is that only one time duration and concentration was used. Additional experiments using longer time durations and various concentrations may be helpful in further evaluating the clinical implications of denture adhesive use as the duration of wear and method of use probably differ from patient to patient.

The four denture adhesives evaluated in this study imparted cytotoxic effects on human gingival fibroblast cells. Staydent showed the highest toxicity. As these denture adhesives demonstrated cytotoxicity to gingival fibroblasts, clinicians should advise the patients not to over-use denture adhesives in terms of amount and time duration. In addition, denture adhesives containing formaldehyde or formaldehyde releasing ingredients should be avoided when possible.

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