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Are Denture Adhesives Safe for Oral Cells?

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Abstract

Purpose: To compare the cytotoxicity of six commercially available denture adhesives on human gingival cells: Poligrip Flavour Free Fixative Cream, Fixodent Pro Duo Protection, Novafix cream, FittyDent, Polident Total Action, and Fixodent Pro Plus Duo Protection.

Material and Methods: Eluates of denture adhesives were brought into contact with human gingival cells and compared to untreated cells (w/o any dental adhesive elute). Cell toxicity was assessed by measuring cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assays), cell morphology (immunofluorescence assays), induction of apoptosis/necrosis and production of reactive oxygen species (ROS) (flow cytometry assays). In addition, the pH of each sample was determined. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

Results: All denture adhesives tested led to a reduction in pH, especially Fixodent Pro Duo Protection and Fixodent Pro Plus Duo Protection. The cell viability assays showed that Fixodent Pro Duo Protection (1:1 72 hours, $p = 3.04 \times 10^{-6}$; 1:2 72 hours, $p = 2.07 \times 10^{-6}$; 1:4 72 hours, $p = 2.04 \times 10^{-6}$) and Fixodent Pro Plus Duo Protection (1:1 72 hours, $p = 2.01 \times 10^{-6}$; 1:2 72 hours, $p = 3.03 \times 10^{-6}$; 1:4 72 hours, $p = 2.02 \times 10^{-6}$) significantly decreased cell viability at all dilutions. Compared to the control group and the rest of the adhesives, Poligrip Flavour Free Fixative Cream (PFF 1:1 72 hours, $p = 2.24 \times 10^{-6}$; 1:2 72 hours, $p = 2.44 \times 10^{-6}$; 1:4 72 hours, $p = 2.04 \times 10^{-6}$) showed a significantly higher cell viability score at all dilutions. Fixodent Pro Duo Protection and Fixodent Pro Plus Duo Protection, both adhesives containing zinc salts in their composition, were responsible for necrosis, and the number of cells was much reduced, with aberrant morphology and pyknotic nucleus. Finally, Fixodent (1:2, $p = 2.04 \times 10^{-6}$, 1:4, $p = 0.00036$; 1:2, $p = 8.82 \times 10^{-6}$, 1:4, $p = 2.30 \times 10^{-6}$) products significantly promoted ROS production in gingival cells.

Conclusions: The results suggest that denture adhesives containing zinc in their composition could be responsible of the decrease of cell viability, ROS production, aberrant cell morphology, and induction of apoptosis and cell death. However, other possible additional cytotoxic factors must be considered. Thus, more studies are necessary to confirm this hypothesis.

Many patients with dentures use denture adhesives daily because they provide retention and stability, improving both function and comfort.[1-3] Moreover, the product is easily available at any pharmacy, with no need for a prescription and at an affordable price.[4] However, the effects that this type of product can have on oral mucosa cells have not been extensively tested, despite the fact that the substance is in contact with the oral mucosa many hours a day, or could even be ingested by the user.[5,6]

Denture adhesives have three main components: those responsible for adhesion, those which act as antimicrobial agents and others that act as fillers. The adhesive substances include karaya gum, gelatin, pectin, methylcellulose, hydroxymethyl cellulose, and carboxymethyl cellulose sodium salt.[3] The main

Table 1 Material tested

Material	Manufacturer	Composition	Lot number
Poligrip Flavour Free	GlaxoSmithKline, Consumer Healthcare SA. Stafford Miller (Ireland) Limited, Clochreane, Youghal Road, Dungarvan, Co Waterford, Ireland.	Calcium/sodium, PVM/MA copolymer, petrolatum, cellulose gum, paraffinum liquidum.	AJ4M
Fixodent Pro Duo Protection	Procter & Gamble Portugal S.A., Qta da fonte, Ed. Álvares Cabral, 2774-527, Paço de Arcos, Portugal.	Calcium/Zinc PVM/MA copolymer, paraffinum liquidum, cellulose gum, petrolatum, silica, CI 15986, menthyl lactate, aroma CI45410, sodium saccharin, limonene, cinnamal eugenol.	61373
Novafix cream	Laboratorios Urgo S.L.U., Florida 29, 20120 Hermani, Guizpuzcoa, España.	Paraffinum liquidum, calcium/sodium, pvm/ma copolymer, cellulose, gum, petrolatum, silica.	ES0004357/171
Fitty® Dent	Fiftydent International GMBH, A-7423 Pinkafeld, Austria	Sodium, carboxymethylcellulose, polyvinylacetate, alcohol denat., triacetin, paraffinum liquidum, silica, aloe vera gel, *Commiphora myrrha* extract.	1082019
Polident Total Action	GlaxoSmithKline, Consumer Healthcare SA. Stafford Miller (Ireland) Limited, Clochreane, Youghal Road, Dungarvan, Co Waterford, Ireland.	Calcium/Sodium, PVM/MA, copolymer, petrolatum, cellulose gum, paraffinum liquidum, tocopheryl acetate, methylparaben, ethylparaben.	S36A
Fixodent Pro Plus Duo Protection	Procter & Gamble Portugal S.A., Qta da fonte, Ed. Álvares Cabral, 2774-527, Paço de Arcos, Portugal	Calcium/Zinc PVM/MA copolymer, paraffinum liquidum, petrolatum, cellulose gum, silica, CI 15985, Menthyl Lactate, Aroma, CI 45410, sodium saccharin, limonene, cinnamal, eugenol.	71636

purpose of the antimicrobials, such as sodium borate, sodium tetraborate, hexachlorophene or propyl hydroxybenzoate, is to prevent the appearance of *Candida Albicans* fungal infections because some studies have shown that the use of denture adhesives increased its growth.[3,7] Finally, the additives that serve as fillers, such as magnesium oxide, in addition to moistening, are intended to impart a pleasant taste for the user (e.g., mint or gaultheria oil).[8,9]

Denture adhesives are sold both as creams and powders.[10] This study was focused on the use of cream format adhesives because they have more bond strength and the time in contact with the oral mucosa is longer than in the case of a powder.[10,11] Also, this format contains cellulose derivatives in its composition such as methylcellulose, hydroxymethyl cellulose or carboxymethyl cellulose to increase its retention properties.[3]

Manufacturers are aware that the product may be ingested, and studies that have been conducted into possible side effects have identified rare reactions, such as nausea, epigastric pain and vomiting.[3] However, we have found no articles regarding the relationship of denture adhesives and the oral cells they come in contact with.

As mentioned above, the composition of denture adhesive, which is intended to enhance denture retention, stability and function,[12] differs from one manufacturer to another. However, there is scarce information on their potential toxicity. Thus, the objective of this study was to compare the cytotoxicity on human gingival fibroblasts of six denture adhesives that are readily available: Poligrip Flavour Free Fixative Cream (PFF), Fixodent Pro Duo Protection (FPDP), Novafix cream (NC), FittyDent (FD), Polident Total Action (PTA), and Fixodent Pro Plus Duo Protection (FPPDP).

Materials and methods

The six commercially available denture adhesives tested and their compositions are shown in Table 1. Only two of them (i.e., FPDP and FPPDP) contain zinc salts. Eluates of these materials were prepared, following the recommendations of ISO 10993–5.[13] For this, 0.2 g of each denture adhesive was immersed in 1 mL of Minimum Essential Medium Alpha (α-MEM; Gibco) and incubated for 24 hours at 37°C. Due to the high viscosity of the materials, three centrifugation steps were performed (10 min at 4000 rpm) until a clear and non-solid extract was obtained. Extracts were collected and diluted in serum-free culture medium (1:1, 1:2, and 1:4), the different tests were carried out at different time periods (24, 48, and 72 hours), to asses both exposure time and concentration, in attempt to mimic the clinical real situations in which patients do not remove their prosthesis or do not clean them properly.

After increasing incubation periods (24, 48, and 72 hours) in a cell culture incubator at 37°C, 5% CO_2, and 95% humidity, the pH of the solution in the culture tube for each sample was measured using a twin pH meter (GLP21+, Crison). Sample size was 10 ml for each eluate and three eluates were used for each denture adhesive. Each eluate was measured every 24 hours for 72 hours.

Human gingival fibroblasts were obtained from impacted tooth extraction ($n = 10$), in accordance with the ethical committee of Murcia University (UM; ID: 2199/2018). All individuals were informed about the research project and signed the informed consent form as per Helsinki Declaration guidelines. The excised gingival tissues were immediately digested for 2 hours at 37°C with 3 mg/ml collagenase I (Sigma-Aldrich) and 4 mg/ml dispase II (Invitrogen). Then, the cells were cultured in basal growth medium (Dulbecco's modified Eagle's medium (Invitrogen)), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Gingival fibroblasts were plated onto 96-well plates at a density of 10^4 cells/well, and incubated at 37°C and 5% CO_2 until confluence. The cell culture medium was removed from each well and replaced by one of the previously described eluates

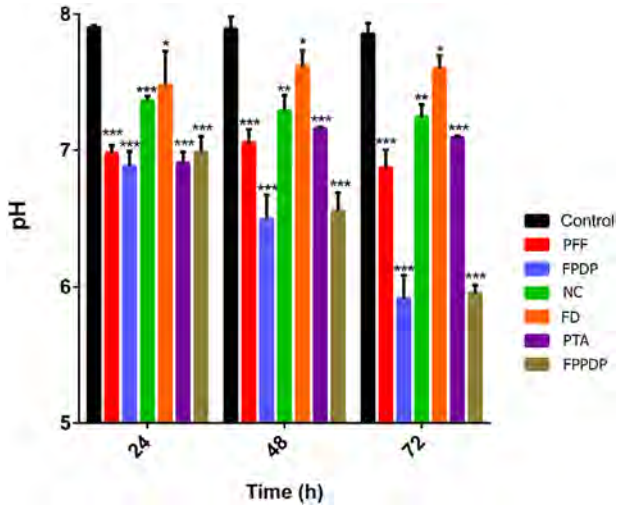

Figure 1 Analysis of pH of the different extracts performed using a twin pH meter. The results are represented as the mean ± standard deviation.

(1:1, 1:2 and 1:4) for each denture adhesive. Six measurements were done for each dilution and for each time (24, 48, and 72 hours), and samples size were 180 µl for each measurement. Cells without extracts served as control. In accordance with the manufacturer's instructions, the MTT reagent (Sigma-Aldrich) was added to the wells and incubated for 4 hours. When the purple precipitate was clearly noticeable, dimethyl sulfoxide (DMSO) (100 µl/well) was added to solubilize the formazan dye. Absorbance in each well was evaluated at 570 nm using a microplate reader (Synergy H1, BioTek) at 570 nm (Abs570).

Confocal analysis was used to analyze changes in the actin cytoskeleton organization of gingival cells, as previously described.[14] Cells were seeded directly on glass coverslips (10-mm^2) in 12-well plates cultured in culture medium containing the undiluted extracts. Then, the cells were fixed with a solution of 4% paraformaldehyde in PBS, washed twice with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 10 min. F-actin cytoskeleton was detected by incubating cells for 1 hour with CruzFluor594-conjugated phalloidin (Santa Cruz Biotechnology). After washings, cells were incubated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) for 1 hours at 37°C for cell nuclei staining. Images were acquired using an AxioImager M2 Zeiss microscope (Carl Zeiss).

The percentages of early and late apoptotic cells were analyzed using an Annexin-V/7-AAD staining kit (BD Biosciences). Briefly, gingival cells were firstly cultured for 72 hours with three different dilutions (1:1, 1:2, and 1:4) of the analyzed denture adhesives. After, cells were detached with trypsin-EDTA and washed twice with PBS. Then, the cells were stained with 5 µl of FITC-conjugated Annexin-V and 5 µl of 7-AAD in 100 µl of 1× Annexin-V binding buffer for 15 min at room temperature. Finally, cells were acquired in a BD LSR Fortessa flow cytometer (BD Biosciences).

To determine the levels of ROS produced by the eluates of the different denture adhesives, gingival cells were resuspended in 1 ml of complete growth medium and stained with 5 µM of CM-H_2DCFDA (Invitrogen) for 30 min at 37°C. For these experiments, three different dilutions (1:1, 1:2, and 1:4) of the analyzed denture adhesives were used. Then, gingival cells were washed twice and analyzed by flow cytometry as above. All flow cytometry determinations were performed in triplicate and repeated three times.

The data obtained were analyzed using Graph-Pad Prism (version 8.1.0, GraphPad Software). Data from results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. *p*-Values <0.05 were considered significant.

Results

After 24 hours of incubation, all denture adhesives tested produced a reduction in the pH of the growth medium, especially FPDP ($p = 8.84 \times 10^{-6}$) (F = 30.82); at 48 hours, FPDP and FPPDP caused a drastic reduction in the pH of the medium (FPDP $p = 6.12 \times 10^{-6}$; FPPDP $p = 5.33 \times 10^{-6}$) (F = 59.07) while FD produced a slight decrease in pH ($p = 0.0489$) (F = 59.07). Finally, after 72 hours of incubation, the pH of the medium containing PFF and PTA was found to be significantly lower than that of the control (PFF $p = 0.0001$; PTA $p = 9.11 \times 10^{-6}$), but higher than those of FPDP and FPPDP (FPDP $p = 7.04 \times 10^{-6}$; FPPDP $p = 6.44 \times 10^{-6}$) (F = 164.8) (Fig. 1).

The metabolic activity of cells exposed to adhesive extracts was analyzed at 24, 48, and 72 hours and at 1:1, 1.2, and 1:4 dilutions. One-way ANOVA of 24 hours: F value = 577.2. One-way ANOVA of 48 hours F value = 827.3. One-way ANOVA of 72 hours: F value = 763.9. FPDP and FPPDP decreased cell viability significantly at all dilutions (FPDP 1:1 72 hours, $p = 3.04 \times 10^{-6}$; 1:2 72 hours, $p = 2.07 \times 10^{-6}$; 1:4 72 hours, $p = 2.04 \times 10^{-6}$; FPPDP 1:1 72 hours, $p = 2.01 \times 10^{-6}$; 1:2 72 hours, $p = 3.03 \times 10^{-6}$; 1:4 72 hours, $p = 2.02 \times 10^{-6}$). Cell viability with PFF was significantly higher at all dilutions compared with the control group and the rest of the adhesives (PFF 1:1 72 hours, $p = 2.24 \times 10^{-6}$; 1:2 72 hours, $p = 2.44 \times 10^{-6}$; 1:4 72 hours, $p = 2.04 \times 10^{-6}$). PTA also produced higher cell viability than the control group, except when undiluted at 48 hours, but the PTA results were worse than those of PFF in this respect. NC led to lower cell viability than the control group, PFF and PTA, but better than FPDP and FPPDP (Fig 2).

After 72 hours of exposure of the cell cultures to the extracts of different denture adhesives, large amounts of well-adhered cells were observed for PFF, PTA, and FD (Fig 3). NC also showed some cells but in smaller quantities. Of particular was that the presence of cells exposed to FPDP and FPPDP was minimal, and the few that were observed had an aberrant morphology and a pyknotic nucleus, reflecting the apoptotic or necrotic status of the gingival cells.

The apoptosis/necrosis rates of gingival cells treated with denture adhesives were analyzed by flow cytometry. After 72 hours of exposure, the number of viable cells was reduced when eluates with the higher concentrations of FPPDP and FPDP were used (data not shown); a comparison between the groups with undiluted extracts gave the following percentages of viable cells: FD > NC > PTA = PFF > FPDP > FPPDP. Using the 1:2 dilutions, the order was: FD > PFF > NC > PTA

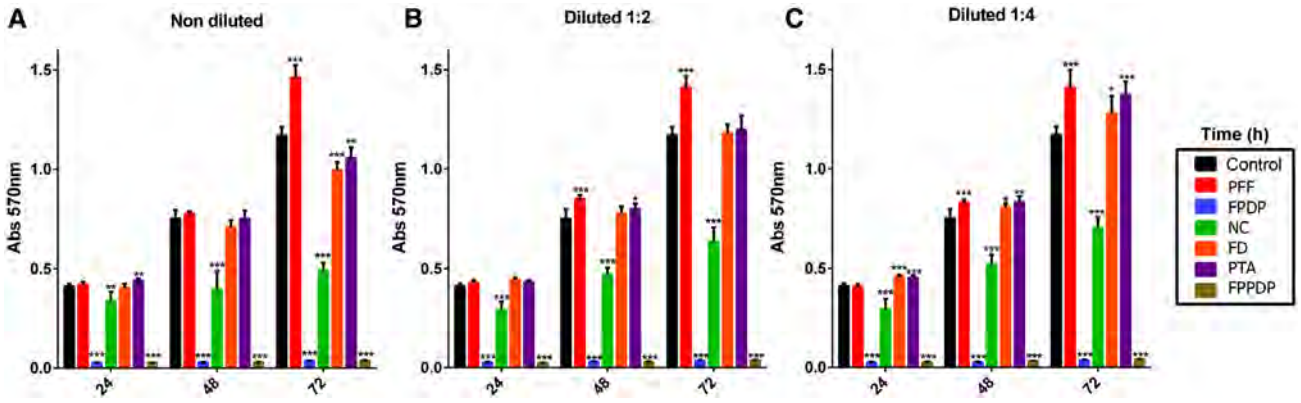

Figure 2 MTT assays. Cell viability of gingival fibroblasts exposed to adhesive extracts for 24 hours. (2A non diluted, 2B diluted 1:2 and 2C diluted 1:4) Absorbance values significantly differed from those of the control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, respectively, by one-way ANOVA and Dunnett's multiple comparisons test).

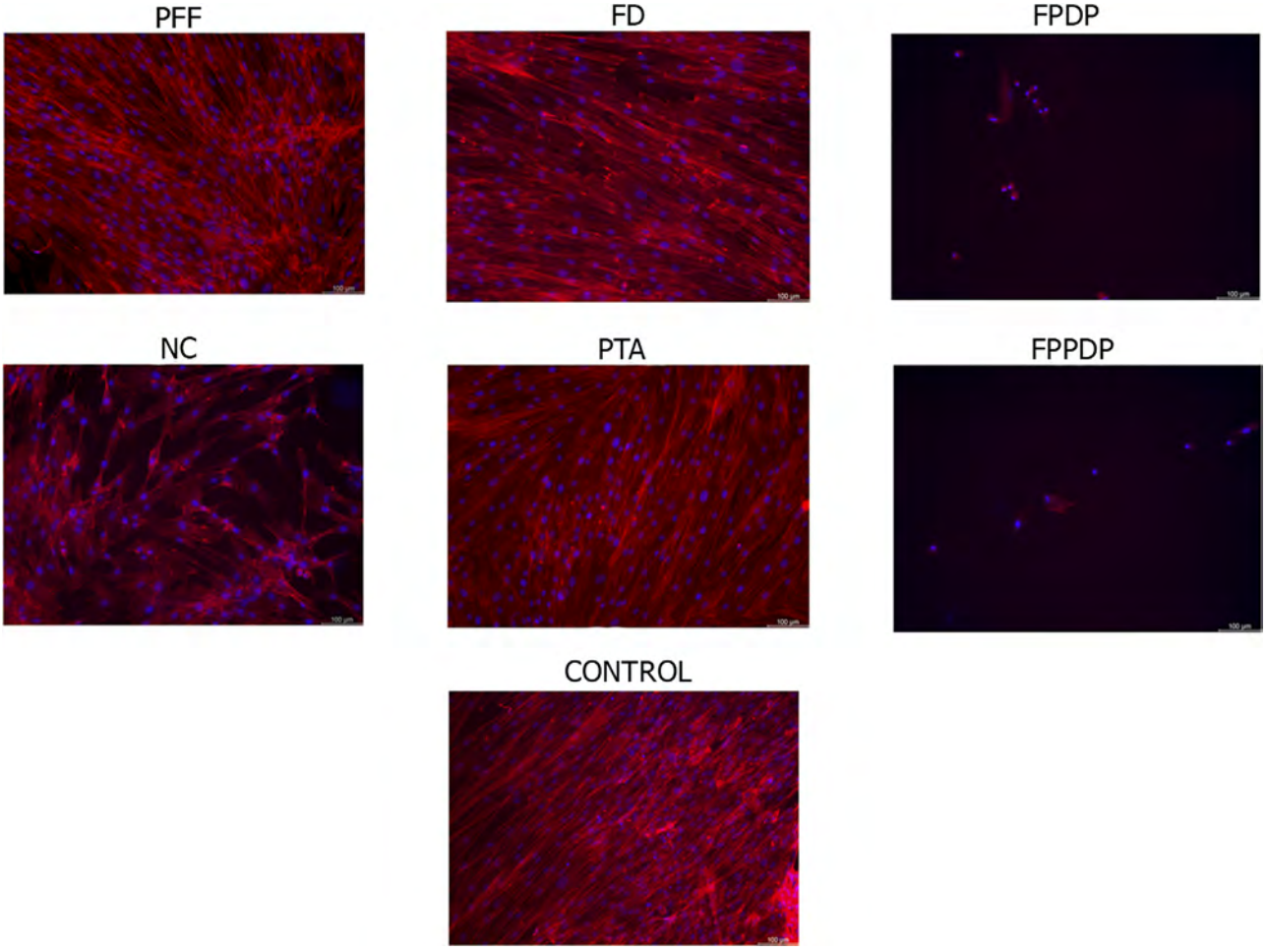

Figure 3 Morphological aspects of human gingival fibroblast cell cultures exposed to extracts of PFF, FPDP, NC, FD, PTA, and FPPDP. Blue fluorescence (DAPI) indicates cell nuclei, and red fluorescence (phalloidin), the actin cytoskeleton. The presence of nuclei and the correct morphology suggest less cytotoxicity. Scale bar = 100 μm.

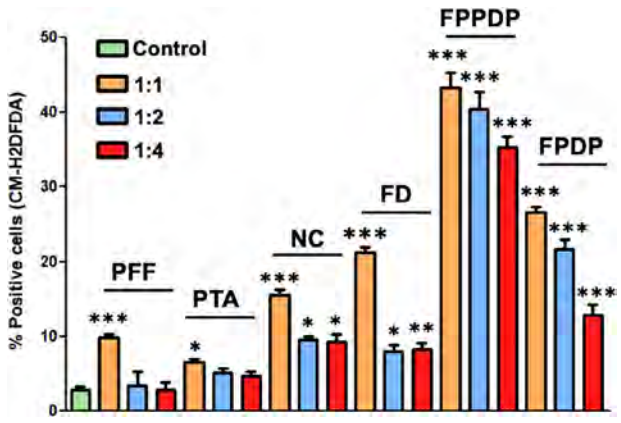

Figure 4 Reactive oxygen species (ROS) production. CM-H_2DCFDA staining was used to evaluate intracellular ROS production in human gingival cells after dental adhesive elutes treatment. Bar graph showing percentages of positive cells for ROS production in each experimental condition are shown.

> FPDP > FPPDP; and using the 1:4 dilutions: FD > PTA > NC > PFF > FPDP > FPPDP (data not shown).

Intracellular ROS production induced by the denture adhesives was also evaluated using the general oxidative stress indicator CM-H_2DCFDA. As shown in Fig. 4, percentages of positive cells for CM-H_2DCFDA staining was significantly higher in all denture adhesives groups (dilution 1:1) compared to control cells. Remarkably, the following serial dilutions of these materials (1:2 and 1:4) induced significantly ROS production, mainly in the FPDP and FPPDP groups (FPDP 1:2, $p = 2.04 \times 10^{-6}$, FPDP 1:4, $p = 0.00036$; FPPDP 1:2, $p = 8.82 \times 10^{-6}$, FPPDP 1:4, $p = 2.30 \times 10^{-6}$).

Discussion

Long-term in vivo studies to investigate potential toxic effects of denture adhesives are limited.[15] In this study, human gingival cells were exposed to six commercial denture adhesives to assess their biological properties. In general, excellent cytocompatibility was observed with PFF and PTA products. Conversely, Fixodent products displayed lower cytocompatibility when compared to the control group and with the other tested materials.

The different compositions of denture adhesives and changes in the pH after exposure to the adhesives exposition in a culture medium is one reason for the different degrees of cytotoxicity observed.[16] The results point to significant modifications on the pH of the culture media following the addition of the denture adhesives. Previous reports have described changes in pH after different periods of incubation,[5, 16] with a reduction below 5.0 in one study.[17] However, de Gomes et al. described different degrees of cytotoxicity without changes in the pH of the culture medium.[18] Also, the presence of the other components such as zinc salts in the composition of Fixodent products could be responsible for the observed cytotoxicity effects. In agreement with our results, other studies using eluates from other dental materials have reported that Zn^{2+} induces cytotoxic effects.[19-21] However, to affirm this conclusion it is necessary to analyze these same products but without the presence of zinc salts in their composition.

In this study, cell viability was evaluated in the presence of denture adhesives using the MTT assay. The test found no statistically significant differences in PFF group, but at 72 hours a statistically significant difference was noticed between PFF, PTA and Fixodent group, with the maximum effect observed in the PFF group. These findings are consistent with previous research showing that L929 showed significantly decreased viability at 12 hours when exposed to Fixodent.[18] However, in another work, three commercial denture adhesives (Polident, Protefix cream, and Proteofix powder) were found to be nontoxic to mouse cells, but significantly reduced the viability of oral cells in a metabolic activity assay. The divergence between the results of these studies may be caused by modifications in the experimental parameters measured, such as cell material contact method, cell type, time points, or the preparation of solid specimens or eluates.[18] Several reports point to differences in cytotoxicity that depended on the cell lines and assay techniques used.[1,4,18] This is why human oral cells were used in this study. Besides, previous reports have mentioned that information in cytotoxicity screening is more useful compared with animal cell lines, and also easier to translate the findings obtained to the clinical practice.

It has been reported that cytotoxic dental materials induce shrinkage of the actin cytoskeleton, pyknotic nuclei (predominantly rounded in shape) and a reduction in adherent cells numbers.[6,18] Cytoskeletal alterations, an important sign of a cytotoxic response, were observed in the presence of FPPDP eluates.[18]

In this study, the denture adhesive extracts in contact with gingival cells had several effects in terms of induction of apoptosis and/or necrosis. FPPDP and FPDP were responsible for necrosis, whereas gingival cells underwent apoptosis after contact with FPDP (dilution 1:4).Previous studies have shown that denture adhesives containing formaldehyde or diazolidinyl urea should be discarded because they cause apoptosis.[6,22] However, there is no evidence related to zinc-containing denture adhesives and induction of cell apoptosis/necrosis.

The different tests were carried out at different time periods (i.e., 24, 48, and 72 hours), as recommended by the ISO regulations, in an attempt to mimic the clinical real situations in which patients do not remove their prosthesis or do not clean them properly.

Previous reports suggested that a high level of ROS production impairs cell function and induces premature senescence in cells.[23,24] Increased ROS production accelerates telomere shortening, causes DNA damage, and is associated with cell death.[25] Our study show that Fixodent products promoted ROS production in gingival cells. It was clear that Zn^{2+} exposure led to mitochondrial dysfunction, the inactivation of glutathione reductase, and stimulation of ROS production.[26] It seems that the decrease in gingival cell numbers following exogenous exposure to Zn^{2+} was due to the Zn^{2+} dissolved in the culture medium or within cells.[27] Thus, denture adhesives containing zinc, FPDP and FPPDP, exhibited higher cytotoxicity than denture adhesives that did not contain zinc in its composition. This study has some limitations, such as the lack of performing an EDX to verify the composition of the materials or to be able to

compare the same material with the presence or not of Zn^{2+}. Furthermore, it would be interesting to carry out more studies with other denture adhesives to compare our current results.

Conclusions

These results suggest that denture adhesives containing zinc in their composition could be responsible of the decrease of cell viability, ROS production, aberrant cell morphology, and induction of apoptosis and cell death. However, other possible additional cytotoxic factors must be considered. Thus, more studies are necessary to confirm this hypothesis.

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