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Antimicrobial and cytotoxic effects of denture base acrylic resin impregnated with cleaning agents after long-term immersion

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ABSTRACT

The coadjutant method for denture cleansing most used by denture wearers is immersion in chemical agents, which are toxic when in direct contact with cells. However, clinically, the contact between these chemical agents and prosthetic tissues does not occur directly, but rather with what remained impregnated into acrylic bases, even after rinsing the disinfected dentures. This study evaluated the antimicrobial and cytotoxic effects of a denture acrylic resin after successive cycles of daily overnight immersion in 1% sodium hypochlorite (1%NaClO) and 2% chlorhexidine digluconate (2%CHX), simulating the periods of 9 months or 1.5 year. Microbiological and cytotoxic assays were performed, respectively, by broth microdilution method (*Candida albicans* or *Staphylococcus aureus*) and MTT assay. Chemical residues of 2%CHX impregnated into the denture acrylic resin had an antimicrobial effect on both immersion periods, which was not observed with those of 1%NaClO. However, residues of 2%CHX were severely cytotoxic to human gingival fibroblasts compared to those of 1% NaClO and acrylic resin (not submitted to the denture cleansers), which were slightly cytotoxic. Even at low concentrations recommended for overnight soaking of removable dentures, the chemical residues of CHX may result in some degree of toxicity to the denture-bearing mucosa after long-term daily immersion.

1. Introduction

Inadequate cleaning of prostheses promotes the accumulation and adhesion of biofilm, resulting in unpleasant odor, wear of artificial teeth and denture base, as well as the occurrence of local infections, especially denture stomatitis, or even systemic diseases (O'Donnell et al., 2016). Denture stomatitis is the most common form of oral candidal infection found on the palate of denture wearers in up to 88% (Aoun and Berberi, 2017; Rivera et al., 2017; Salerno et al., 2011; Webb et al., 2005). The complex, multispecies nature of biofilms involved in denture stomatitis, mainly demonstrated by the frequent microbial association of *Candida albicans* and *Staphylococcus aureus* (Baena-Monroy et al., 2005; Chopde et al., 2012; Pereira et al., 2013), requires procedures for denture cleansing that are effective in inhibiting both fungi and bacteria (Arruda et al., 2017).

Cleansers are well accepted by removable denture wearers, since they are easy to handle, affordable, present good cost-benefit relationship, and are also adequate for institutionalized individuals (de Andrade et al., 2012; Webb et al., 2005). Among the solutions employed for cleansing and disinfection of complete dentures, emphasis is given to sodium hypochlorite and chlorhexidine digluconate, which present fungicidal action and are also effective to control the denture biofilm (de Andrade et al., 2012; Pellizzaro et al., 2012), and therefore are recommended as coadjutant methods for the treatment of denture stomatitis (Arruda et al., 2017; Banting and Hill, 2001; Webb et al., 2005).

Despite the beneficial properties, some adverse effects have been related to the extended use of these cleansers. Chlorhexidine solutions employed for long periods may cause staining of denture bases (Asad et al., 1993) and tongue epithelium (Budtz-Jörgensen and Löe, 1972), besides reducing the hardness of artificial acrylic teeth (Campanha et al., 2012). Immersion in sodium hypochlorite for long periods may be corrosive to metals and influence the color stability, flexural strength, roughness, and hardness of denture base acrylic resins (Davi et al., 2010; Felipucci et al., 2011; Hong et al., 2009; Paranhos et al., 2013).

Other concern related to denture cleansers refers to the potential cytotoxic effects of these solutions to the oral tissues. Sodium hypochlorite solutions are moderately toxic to several human cells, including dermal and oral epithelium cells (Hidalgo et al., 2002; Sagripanti and Bonifacino, 2000). Even at concentrations lower than used in clinical

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practice, chlorhexidine solutions caused significant toxic effects to several cells, such as dermal fibroblasts (Hidalgo and Dominguez, 2001), oral epithelium cells (Eren et al., 2002) and lymphocytes (Arabaci et al., 2013). It has also been reported that chlorhexidine formulations for clinical use caused genotoxicity and cytotoxicity to oral tissues and cells (Arabaci et al., 2013; Giannelli et al., 2008; Hidalgo and Dominguez, 2001; Li et al., 2014; Ribeiro et al., 2004; Sagripanti and Bonifacino, 2000; Shetty et al., 2014). However, the toxicity effects of previous studies should be carefully analyzed when considering the use of these chemical disinfectants as denture cleansers, since the solutions were tested in direct contact with human or animal cells (Arabaci et al., 2013; Ghabanchi et al., 2013; Giannelli et al., 2008; Hidalgo and Dominguez, 2001; Hidalgo et al., 2002; Li et al., 2014; Mirhadi et al., 2014; Ribeiro et al., 2004; Sagripanti and Bonifacino, 2000). On the other hand, clinically, the contact between chemical agents and denture supporting tissues does not occur directly, but rather with what remained impregnated into acrylic bases, even after rinsing the disinfected dentures.

This study evaluated, in the long term, the antimicrobial and cytotoxic effects of sodium hypochlorite and chlorhexidine digluconate solutions that remain impregnated into the denture base acrylic resin after successive cycles of daily overnight immersion. The tested hypotheses were that the chemical residues of cleansing agents impregnated into the acrylic resin would result in inhibition of pathogens associated with denture stomatitis, and in cytotoxic effects to the human gingival cells.

2. Material and methods

2.1. Fabrication of specimens and protocol of immersion in denture cleansers

Specimens (n = 186; 10 × 1 mm) were produced after manipulation, proportion, and polymerization of the denture base acrylic resin (Lucitone 550®, Dentsply Ind. Com. Ltda., Petrópolis, RJ, Brazil) in a water bath according to manufacturer's instructions (73 °C for 90 min and 100 °C for 30 min). After excess removal, the surfaces of specimens were manually ground with 180-grit silicon carbide paper to achieve a surface roughness of ± 2 μm (Surftest SJ-301, Mitutoyo Co., Tokyo, Japan), simulating the mean roughness of the internal surface of a complete denture (da Silva et al., 2011). Initially, the specimens were sterilized in microwave for 3 min at 650 W(Altieri et al., 2012; Altieri et al., 2013; Pellizzaro et al., 2012) and stored in sterile distilled water at 37 ± 1 °C for 50 ± 2 h (ISO, 1998). Then, they were submitted to three daily disinfection cycles of 8 h each in one of the tested denture cleansers: 1% sodium hypochlorite (1%NaClO); 2% chlorhexidine digluconate (2%CHX) (Pharmácia Specifica Ltda, Bauru, SP, Brazil); and sterile distilled water (solution control-H_2O) for periods of consecutive 91 days (T91) or 183 days (T183), respectively simulating 9 months of 1.5 year of daily overnight immersion performed by the patient (da Silva et al., 2011; Paranhos et al., 2013; Pisani et al., 2012a; Pisani et al., 2012b). The solutions were replaced once a day during the experimental periods (Pisani et al., 2012a).

2.2. Residual antimicrobial effect of denture cleansers

The antimicrobial effect of denture cleansers impregnated into the acrylic resin was assessed by the broth microdilution method to determine the minimal inhibitory concentrations (MICs). The specimens of each solution and different immersion periods were immersed in specific culture media containing the inoculum of *C. albicans* (YEPD, Clontech laboratories Inc., Mountain View, CA, USA) or *S. aureus* (BHI, DIFCO TM, Becton Dickson Co., San Jose, CA, USA) (Baena-Monroy et al., 2005; Chopde et al., 2012; Pereira et al., 2013) spectrophotometrically standardized at an optical density at 600 nm (Altieri et al., 2012; Altieri et al., 2013; Spiechowicz et al., 1990) to a final concentration of 1×10^7cells/mL (Altieri et al., 2012; Altieri et al., 2013; Pellizzaro et al., 2012). Wells containing only the fungal/bacterial inoculum or only the culture media were used as positive and negative microbial controls, respectively. The microplates were incubated aerobically at 37 °C for 24 h, 7, and 14 days. The microbial viability was analyzed by turbidity determined by absorbance levels at 600 nm (Spiechowicz et al., 1990).

Since the same specimen was tested at different incubation periods, each experimental condition was designed according to the solution, immersion time, and pathogen. Three independent experiments were performed, with each condition tested at least in triplicate. In each experiment, 5 specimens of each experimental condition were tested. Thus, a total of 180 specimens was obtained.

The MICs in the different periods were determined by calculating the percentage of microbial viability from the optical densities obtained from wells containing the specimens compared to the positive microbial control (100% of viability). Thereafter, the percentages of cell death were calculated by subtracting the percentages of viability of 100% extracts. The percentages of microbial reduction were obtained by the optical densities of groups presenting residual effects of denture cleansers (experimental) in relation to the control group (without inhibition of microbial growth), according to the following formula:

$$\%\text{reduction} = \frac{(\text{Abs } 600 \text{ nm})\ \text{control} - (\text{Abs } 600 \text{ nm})\ \text{experimental}}{(\text{Abs } 600 \text{ nm})\ \text{control}} \times 100$$

2.3. Analysis of residual cytotoxic effect of denture cleansers

The *in vitro* analysis of the cytotoxic effect of the chemical residues of denture cleansers impregnated into and released by the specimens was performed by the MTT colorimetric assay [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] on human gingival fibroblasts (L929).

Initially, the specimens were aseptically rinsed in sterile distilled water to eliminate the possible excesses of denture cleansers (Campanha et al., 2012; Paranhos et al., 2013). To achieve the extracts (ISO, 2009), three specimens immersed in each denture cleanser (CHX, NaClO, and H_2O) for one of the experimental periods (T91 and T183) were placed in 3 mL of Eagle medium supplemented with 7.5% Fetal Bovine Serum containing 80 μg/mL of gentamicin (Sigma-Aldrich Inc., St. Louis, MO, USA). The specimens were incubated at 37 °C for 24 h for diffusion of possibly toxic substances to the culture medium (extracts). To investigate the possible cytotoxic effects of the resin components, three specimens, immediately after fabrication and not submitted to disinfection, were tested in the same conditions described above (acrylic resin control).

Cells were seeded at a density of 1×10^5cells/mL in Eagle medium supplemented with 7.5% Fetal Bovine Serum containing 80 μg/mL of gentamicin, maintained in a humidified incubator at 37 °C under a 5% CO_2 and 95% air atmosphere (Fanem®, São Paulo, SP, Brazil). For the cytotoxicity assay, 100 μL of cell suspension were placed in each well in a 96-well culture plate, incubated at 37 °C in an oven with 5% CO_2 for 24 h. After this period, the culture medium was discarded, and cells adhered to the bottom of the plate were maintained. Then, fresh culture medium (50 μL) was added in each orifice in the plate combined with 50 μL of specimens extracts and submitted to incubation for 24 h in an oven with 5% CO_2. Following, 10 μL of MTT (5 mg/mL) were added to each plate well, which remained incubated for 4 h at 37 °C for formation of violet formazan crystals stored in the cytoplasm of cells, resulting from mitochondrial activity. Thereafter, 100 μL of MTT solubilization solution (DMSO) were added to each plate well, which were gently shaken to dissolve the formazan crystals, allowing analysis of cell viability by the mitochondrial activity in a spectrophotometer at 500 nm (Jorge et al., 2004). Wells containing only fresh culture medium (100 μL) in the same aforementioned conditions were used as negative control. Therefore, the experimental conditions were as