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RESEARCH AND EDUCATION

Cytotoxic potential of denture base and reline acrylic resins after immersion in disinfectant solutions

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ABSTRACT

Statement of problem. The daily immersion of dentures in disinfectant solutions can cause the incorporation of toxic substances in the acrylic resins, and studies evaluating the cumulative effect of disinfectant solutions on cell culture are lacking.

Purpose. The purpose of this in vitro study was to evaluate the cytotoxic potential of cell cultures of denture base and reline acrylic resins after immersion in disinfectant solutions.

Material and methods. Disk-shaped specimens (14×1.2 mm) were obtained and divided into groups (n=9) according to the disinfectant solutions (distilled water [control], 2% chlorhexidine digluconate, 3.8% sodium perborate, 0.5% sodium hypochlorite, and apple vinegar) and to the storage period (0, 1, 3, and 6 months). Solutions were changed daily. After the different storage periods, specimens were immersed in culture medium for 24 hours, and extracts were obtained. Human keratinocytes were cultivated, and the cellular metabolism was evaluated by using Alamar Blue. Data were submitted to 3-way analysis of variance and Games-Howell post hoc tests (α=.05).

Results. Both of the acrylic resins tested showed similar biocompatibility properties after immersion in different solutions (P=.400). Immersion in distilled water, 3.8% sodium perborate, and 0.5% sodium hypochlorite did not affect the cellular metabolism of the keratinocytes (P>.05), regardless of the immersion period and the type of acrylic resin (P>.05). Immersion in 2% chlorhexidine digluconate or apple vinegar resulted in high cytotoxicity over time, until the third month (P<.05), after which no changes were observed (P>.05).

Conclusions. The type of acrylic resin (base or reline) had no significant effect on the viability of cells. Vinegar and chlorhexidine digluconate solutions increased in cytotoxic effect over time, and were strongly cytotoxic after 6 months of immersion. Sodium perborate and sodium hypochlorite were noncytotoxic in all periods of time tested. (J Prosthet Dent 2018;120:155.e1-e7)

Denture bases and/or reline materials present surface irregularities and microporosities that facilitate fungal adherence and colonization through the formation of biofilms on the prosthesis surface. In this mode of growth, *Candida* spp. proliferate as a community of adherent cells covered in an extracellular matrix.[1] This type of biofilm is resistant to several drug classes and is capable of withstanding high antifungal concentrations.[2,3] The clinical relevance of the presence of a biofilm on the acrylic resin surface is that the denture can provoke palatal mucosa injury, facilitating denture stomatitis. In addition, poor oral mucosa or denture hygiene allows the adhesion of debris to the surfaces, which also can be a potential source of contamination.[4] Rigorous hygiene habits are needed for both the denture surface and the oral mucosa to maintain oral health and prevent the appearance of denture stomatitis in partial or complete denture users.

Several denture stomatitis treatments have been reported, with topicalor systemic use of antifungal drugs being the most common.[5] However, the therapeutic effect of antifungal drugs is limited because of factors such as the appearance of fungal resistance,[3] toxicity,[6] the diluent effect of saliva, and movements of the tongue, which reduce the antifungal agents to

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Clinical Implications

The daily immersion of dentures in some disinfectant solutions increased the cytotoxic effect of the acrylic resins over time. Results should be considered in choosing the immersion solution for disinfection of removable dentures.

sub-therapeutic concentrations[7] and reduce their effects on biofilms colonizing the acrylic resin surfaces.[8] Thus, cleaning and exchanging the dentures are also essential for the resolution of denture stomatitis. In addition, interrupting the use of the denture may reduce the mucosal inflammation in patients with denture stomatitis.[9]

The prosthesis can be cleaned mechanically, chemically, or with a combination of methods.[10] The efficacy of the brushing mechanical method is limited, because the intaglio surface of the prosthesis is irregular and porous.[11] Moreover, elderly patients often lose manual dexterity.[10] Thus, the dentures should be cleaned chemically, such as by immersion in disinfectant solutions.[12] Sodium hypochlorite and chlorhexidine digluconate can inactivate bacterial and fungal biofilms, including those formed by *Candida* spp. and methicillin-resistant *Staphylococcus aureus*.[13] Sodium perborate at 3.8% (active ingredient of Corega Tabs) has antiseptic properties and interferes in the microorganism's metabolism.[14] Studies have also reported the antimicrobial effects of vinegar.[15] Apple vinegar contains a lower concentration of acetic acid (5%) than white wine vinegar (24%) or lemon vinegar (37%).[16] According to Mota et al,[17] apple cider vinegar showed antifungal properties against *Candida* spp., thus representing a possible therapeutic alternative for patients with denture stomatitis.

Daily immersion of the dentures in disinfectant solutions can cause the incorporation of toxic substances into the acrylic resin, which can be released in the oral environment and, consequently, provoke irritation or allergic reaction of the oral mucosa.[18] Other acrylic resin compounds, such as methyl methacrylate, formaldehyde, methacrylic acid, and benzoic acid, promote cytotoxicity to different degrees.[19-22] However, studies evaluating the cumulative effect of disinfectant solutions on denture base and reline materials are lacking. Therefore, the purpose of this in vitro study was to evaluate the cytotoxic potential of denture base and reline acrylic resins after immersion in disinfectant solutions on cell cultures. The null hypothesis tested was that no significant differences in the cytotoxicity of acrylic resin would be found after long-term exposure in the different disinfectant solutions.

MATERIALS AND METHODS

Denture base and reline acrylic resins (Table 1) were selected for this study because, owing to differences in their chemical compositions and water sorption characteristics, they are affected differently by water immersion.[23] Disk-shaped specimens (14-mm diameter and 1.2-mm thickness) of both resin types were made. Denture base resin (Vipi Wave; Vipi Produtos Odontológicos) specimens were prepared by using a cast mold that was sandwiched between 2 glass plates and embedded in a denture flask with dental stone (Herodent; Vigodent). After the dental stone had set, the flask was opened, and the circular opening of the metal mold was filled with the denture base resin manipulated according to the manufacturer's instructions. The flask was placed under a 1.5-ton hydraulic press for 30 minutes. Exposure to microwaves at a maximum power of 800 W was used to polymerize the acrylic resin for 10 minutes at 20% power and 4 minutes at 60% power. Reline resin (Tokuyama Rebase Fast II; Tokuyama Dental Corp) specimens were prepared according to the manufacturer's instructions. The resin was inserted into the circular opening of the metal mold and sandwiched between 2 glass plates. Manual pressure was applied until the material was completely polymerized. Excess denture base and reline resins were removed by using a tungsten carbide bur (Maxi-Cut; Malleifer SA), and the specimens were stored for 48 hours in distilled water to release the residual monomer.[24]

After 48 hours, the specimens were divided into groups (n=9) according to the type of disinfectant solution: distilled water (control group), 2% chlorhexidine digluconate, 3.8% sodium perborate, 0.5% sodium hypochlorite, and apple vinegar. The specimens were stored for 0, 1, 3, and 6 months, and the solutions were changed daily. During each day of the experiment, all specimens were immersed for 8 hours in distilled water or disinfectant solutions and then for 16 hours in distilled water, simulating night disinfection and daily use of the prosthesis. After each immersion period, the specimens were rinsed in water to remove excess disinfectant solution. Subsequently, they were disinfected using ultrasound for 20 minutes and ultraviolet light in a laminar flow for 20 minutes on each side to eliminate microorganisms.[19]

Eluates were obtained from the specimens for the cytotoxic analysis of the incorporated and released substances from the acrylic resins. For this step, 3 specimens from each experimental group were placed in vials with 3 mL of Dulbecco's modified Eagle medium (DMEM) and incubated for 24 hours at 37°C and 5%CO_2.[25] Human keratinocytes (HaCaT line) were cultivated in DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and 1% antifungal

Table 1. Denture base acrylic resins tested

Material	Composition		Powder:Liquid Ratio	Polymerization Cycle	Lot Number
	Powder	Liquid			
Tokuyama Rebase Fast	PEMA	AAEM and 1,9-nonanediol dimethacrylate	2.1g/1.0 mL	5.5 min at room temperature	225EZ3
Vipi Wave	PMMA, benzoyl peroxide, pigments	MMA, EGDMA, inhibitor	2.15g/1.0 mL	10 min at 20% power, followed by 4 min at 60% power	75643

AAEM, 2-acetoacetoxy (ethyl) methacrylate; EDGMA, ethylene glycol dimethacrylate; MMA, methyl methacrylate; PEMA, poly(ethyl methacrylate); PMMA, poly(methyl methacrylate).

antibiotic solution with 10 000 units•mL^{-1} penicillin G, 10 000 units•mL^{-1} streptomycin, and 25 units•mL^{-1} amphotericin. The cells were cultivated in 75-cm^2 cell culture flasks containing a filter that allowed passage of CO_2. The plates were kept at 37°C in an atmosphere of 5% CO_2 in a 95% air and humidity controlled environment. Total viable cells were counted in a Neubauer chamber (New Optics) by adding Trypan blue dye and plating in 1.0×10^4 cells/well in a sterile 96-well plate. Subsequently, the cells were incubated in 5% CO_2 at 37°C for 24 hours. After this period, 100 μL of eluates containing the substances released from the specimens were added in each well of the 96-well plate containing cells and placed inside an incubator containing 5% CO_2 at 37°C for 24 hours. Four compartments of the plate were used (analysis in quadruplicate) for each experimental group. Four wells received only 100 μL of fresh culture medium (DMEM) supplemented with 10% FBS and 1% antibiotic antimycotic solution (negative control group). After 24 hours of incubation, 20 μL of Alamar Blue (Sigma Aldrich)[26-30] diluted in 100 μL of culture medium (10% of total medium volume, 200 μL) was added in each well. Plates were then incubated in 5% CO_2 at 37°C for 4 hours, and the specimens' fluorescence was measured (Fluoroskan Ascent FL; Lab Systems) with filters of 544 nm emission and 590 nm transmission. Additionally, pH measurements of all disinfectant solutions were carried out by using a digital pH meter (model Q400AS; Quimis).

The variable of interest was the fluorescence value obtained from the Alamar Blue test to evaluate cell metabolism after all experimental conditions. The values were converted to a percentage of the negative control group.[31] To qualify the cytotoxic effect of the disinfectant solutions tested, fluorescence values from each experimental group were compared with the fluorescence values obtained from the negative control group (100% viability) and were ranked as follows[27]: 0, not cytotoxic (inhibition was 25% lower than control group); 1, slightly cytotoxic (inhibition was between 25% and 50% in comparison with control group); 2, moderately cytotoxic (inhibition was between 50% and 75% in comparison with control group); and 3, strongly cytotoxic (inhibition was 75% higher than that of the control group).

Percentage data were analyzed by using 3-way analysis of variance (ANOVA) followed by the Games-Howell test for post hoc analysis, which is used when there was heterogeneity of variance in the data (α=.05). The 3 factors considered were disinfectant solutions (distilled water; 2% chlorhexidine digluconate, 3.8% sodium perborate, 0.5% sodium hypochlorite; and apple vinegar), acrylic resins (Vipi Wave and Tokuyama Rebase Fast II), and periods of time (0, 1, 3, and 6 months). All tests were performed using statistical software (IBM SPSS Statistics v21; IBM Corp) (α=.05).

Table 2. Summary of three-way ANOVA

Effect	*df*	Mean	F	*P*
Acrylic resin	1	12.96	0.72	.400
Period of time	3	8211.91	453.83	<.001*
Disinfectant solution	4	23095.56	1276.38	<.001*
Acrylic resin×period of time	3	15.24	0.84	.475
Acrylic resin×disinfectant solution	4	16.98	0.94	.446
Period of time×disinfectant solution	12	2822.02	155.96	<.001*
Acrylic resin×period of time×disinfectant solution	12	5.28	0.29	.989
Residue	80	18.09		

ANOVA, analysis of variance. *Significant at 5% level.

RESULTS

Results of the qualitative analysis of the cytotoxicity of the disinfectant solutions showed that, regardless of the resin type, the solutions were ranked as having no cytotoxicity or slight or strong cytotoxicity. The cell viability after immersion in water (percentage of viable cells ranged from 94.2% to 98.6%), sodium perborate (percentage of viable cells ranged from 89.9% to 96.1%), and sodium hypochlorite solutions (percentage of viable cells ranged from 85.8% to 96%) was higher than 85% at all times tested, so these 3 solutions were classified as not cytotoxic (score, 0). Vinegar and chlorhexidine digluconate solutions showed different cytotoxic effects according to the periods of time. After the first month of immersion, the cell viability after immersion in vinegar and chlorhexidine digluconate ranged from 51.8% to 56.5% and were classified as slightly cytotoxic (score, 1). From the 3rd month until the end of 6 months of immersion in these solutions, there was a great reduction in cell viability, which was, generally, 95% lower than for the control group. Therefore, at 3 and 6 months, vinegar and chlorhexidine digluconate were ranked as strongly cytotoxic (score, 3).

Table 3. Viable cells for resin types in accordance with disinfectant solutions and storage times, mean ±standard deviation (%)

	Storage Time (mo)			
Disinfectant Solution	**0**	**1**	**3**	**6**
Water	94.7 ±3.1^{Aa}	98.2 ±1.1^{Ba}	95.3 ±2.5^{Ba}	94.9 ±3.7^{Ba}
Perborate	92.9 ±4.3^{Aa}	95.7 ±1.4^{Ba}	95.1 ±1.6^{Ba}	93.0 ±4.8^{Ba}
Hypochlorite	91.2 ±2.4^{Aa}	95.0 ±5.1^{Ba}	90.0 ±5.0^{Ba}	86.5 ±6.5^{Ba}
Vinegar	86.9 ±4.7^{Ac}	54.4 ±4.5^{Ab}	4.5 ±0.8^{Aa}	3.6 ±1.6^{Aa}
Chlorhexidine	85.6 ±4.8^{Ac}	52.5 ±4.2^{Ab}	4.4 ±0.6^{Aa}	4.2 ±2.5^{Aa}

Horizontally, identical superscript lowercase letters denote no significant differences among groups (Games-Howell test, *P*>.05). Vertically, identical superscript uppercase letters denote no significant differences among materials (Games-Howell test, *P*>.05).

The summary of the 3-way ANOVA is shown in Table 2. These results indicated no significant differences in the effect of the 2 acrylic resins tested on the percentage of viable cells (*P*=.400). Three-way ANOVA revealed significant effects on the percentage of viable cells for the periods of time (*P*<.001) and disinfectant solutions (*P*<.001), as well as a significant interaction effect between these 2 factors (*P*<.001). Results from the Games-Howell post hoc analysis are listed in Table 3. These results showed that, regardless of the acrylic resin type, the percentage of viable cells after immersion in vinegar and chlorhexidine digluconate declined significantly (*P*<.05) over time, until the third month, after which no changes were observed. Although no significant differences were found between vinegar and chlorhexidine digluconate (*P*>.05), the cytotoxicity of both disinfectant solutions was significant higher (*P*<.05) than that observed for distilled water, sodium perborate, and sodium hypochlorite. Immersion of the specimens in distilled water, sodium perborate, and sodium hypochlorite did not affect the cellular metabolism of the keratinocytes, regardless of the immersion period (*P*>.05) and the type of acrylic resin (*P*>.05). Also, no significant differences were found among these 3 solutions (*P*>.05).

The pH values of the disinfectant solutions are presented in Table 4. Chlorhexidine digluconate showed an acidic pH of 3.9 and a pH of 2.8 in apple vinegar, whereas distilled water, sodium perborate, and sodium hypochlorite had pH values higher than 5.0.

DISCUSSION

This study evaluated the cytotoxic effects of immersing 2 acrylic resins in 4 chemical solutions commonly used to clean and disinfect dentures. The null hypothesis, that no significant differences would be found in the cytotoxicity of acrylic resin after long-term exposure in these different disinfectant solutions, was partially accepted. Results showed that the type of acrylic resins had no significant effect on the viability of cells. Acrylic resin polymerization is essential for optimization of the physical and biological properties of these materials because it allows the conversion of monomers into polymers.[24,31,32] However, differences in the composition of the acrylic resins are more important than the polymerization method in defining their cytotoxicity.[33] In this context, cross-linking agents can be added to acrylic resins to improve the physical and biological properties of the material. In this study, the hard reline resin used is composed of a cross-linked polymer, 2-acetoacetoxy (ethyl) methacrylate,[34] which could explain the results.

Table 4. pH values of disinfectant solutions tested

Disinfectant Solution	pH
Distilled water	7.2
Chlorhexidine digluconate 2%	3.9
Sodium perborate 3.8%	6.7
Sodium hypochlorite 0.5%	5.1
Apple vinegar	2.8

Previous studies have shown that denture base and reline acrylic resins are considered toxic because they inhibit cell proliferation.[35,36] This cytotoxicity could be explained by the release of some compounds of the resins in the culture medium during preparation of the eluates. In the present study, at time zero, none of the groups was cytotoxic, as all experimental conditions evaluated showed a percentage of viable cells higher than 80% compared with the control group, regardless of the type of solution or acrylic resin (*P*>.05). This low cytotoxicity of both resins could be explained by the fact that all specimens were stored in distilled water for 48 hours before immersion in the disinfecting solutions. The storage period was important because the potentially cytotoxic substances from the resins were previously released and did not interfere with the disinfectant solutions. These results agree with those of Jorge et al,[22] who evaluated the cytotoxicity of denture base acrylic resins with and without water storage before eluate preparation and showed that, after 24 hours of water immersion, the resins were considered noncytotoxic.

Different time periods were also considered in the analysis of the cytotoxicity of the materials immersed in disinfectant solutions. Results revealed significant effects for the periods of time (*P*<.001) and disinfectant solutions (*P*<.001) on the percentage of viable cells, as well as a significant interaction effect between these 2 factors (*P*<.001). After 1 month of storage of resins in 2% chlorhexidine and apple vinegar, the cell metabolism of the keratinocytes was reduced to 45% in comparison with cells in the control group, and the extracts were slightly cytotoxic. This toxic effect increased over time, and after 3 months of storage, the

extracts were strongly cytotoxic, reducing the cell metabolism by 95%. In fact, these solutions almost completely inhibited cellular metabolism after 6 months of immersion.

The toxicity of a residual disinfectant can be influenced by factors such its bioavailability after absorption, the route by which residual disinfectant enters the body, the relative toxicity of each disinfectant, and the pH value of the solutions. According to Ryu et al,[37] the toxicity of the residual disinfectant is related not only to poor rinsing but could result from the disinfectants that were absorbed and, consequently, released from the medical devices or materials. The effects of chlorhexidine on a variety of mammalian cells have proved that this solution is a toxic agent at doses similar to or lower than those introduced into the oral cavity.[38-40] The cytotoxicity of apple vinegar can be associated with toxic compounds like peracetic acid and polyphenols.[41] Nakamura et al[42] concluded that apple vinegar caused cell death of keratinocytes after contact for 72 hours because of the release of polyphenols. Another aspect is that the organic acids in vinegar, mainly acetic[43] and peracetic acids,[44] are able to pass into cell membranes leading to cell death[43] and decreased cell viability.[42]

These results can be also explained by the pH of both solutions, as 2% chlorhexidine and apple vinegar have the lowest pH values (3.9 and 2.8, respectively). Acidic pH may have favored the surface degradation of the acrylic resin and, consequently, facilitated the diffusion process of the toxic compound from the acrylic specimens to the culture medium during eluate preparation. Also, water penetrates the matrix and expands the space between polymer chains, allowing unreacted monomers and other toxic substances to diffuse outwardly.[45] These results are in accordance with those of Koda et al,[45] who demonstrated that the residual monomer concentrations increased with a decrease in pH. At maximum leaching, however, the concentration of residual monomer at pH 4.0 was 1.5 times that at pH 6.8. Similarly, Lefebvre et al[31] demonstrated that the pH of the environment affects the amount of cytotoxic substances that leach out of resin. As some compounds act as solvents on the resin surface and are potential sources of damage, the specimens immersed in these solutions were more likely to release potentially toxic substances to the cells. Because the disinfectants were exchanged daily, the specimens were daily exposed to low pH solutions. This maintained the conditions of degradation and the release of toxic components, explaining the increase in toxicity over time, which is in accordance with previous investigations.[33,37]

In comparison with chlorhexidine digluconate and apple vinegar, the other disinfectant solutions were significantly less toxic to the cells ($P<.05$). The storage of specimens in 3.8% sodium perborate and 0.5% sodium hypochlorite did not affect the keratinocytes cellular metabolism, regardless of the immersion period ($P>.05$) and the type of acrylic resin ($P>.05$), with no significant differences between these 2 solutions and distilled water ($P>.05$). Thus, these solutions were considered noncytotoxic from baseline to 6 months, showing at least 85% cell viability in comparison with the control. These results disagree with those of studies demonstrating that sodium hypochlorite and sodium perborate had a negative effect on cell viability in different concentrations.[39,46-48] However, in those previous studies, the toxicity of the disinfectants was evaluated by using the solutions in direct contact with the cells. As explained, this was not our goal. Denture base or reline acrylic resins can absorb and then release compounds in the oral cavity after immersion in disinfectant solutions, so in the present study, acrylic resin specimens were immersed in the solutions for different periods. They were then rinsed in water to remove any excess of disinfectant solution before the cytotoxic assays were performed. Another explanation of the good results obtained with sodium perborate and sodium hypochlorite is their pH values (6.7 and 5.1, respectively), which were similar to that obtained from distilled water (7.2).

In summary, the results of this study in relation to the cytotoxicity of 2% digluconate chlorhexidine and apple vinegar can be explained by 2 primary mechanisms. One mechanism is that absorbed agents from disinfectants are released and negatively impact the cells.[49] The other mechanism is that degradation of the acrylic resins releases toxic byproducts.[32] In addition, it is possible that an interaction of both mechanisms has occurred. Brozek et al[50] showed that dental materials, when placed in disinfectants, produce several chemical compounds such as monomers, plasticizers, and others. This study presented relevant information about the cytotoxicity of 2 commercial brands of denture base and reline acrylic resins after different storage periods in different disinfectant solutions. However, future studies are needed to evaluate other brands of acrylic resins and to investigate whether there is any variation similar to that found here. Furthermore, some limitations should be considered, as the cytotoxicity analysis of cell monolayers. Thus, the use of reconstituted tissues and in vivo studies may complement the results obtained in this investigation.

CONCLUSIONS

Based on the findings of this in vitro study, the following conclusions were drawn:

1. Both denture base and reline acrylic resins showed good biocompatibility after immersion in different

solutions for all periods of time tested, with no significant differences between them.

2. The immersion of denture base and reline acrylic resin specimens in 2% digluconate chlorhexidine and apple vinegar decreased the keratinocytes cell metabolism after 1 month of storage.
3. Extracts were intensely cytotoxic after 3 and 6 months of immersion in 2% chlorhexidine digluconate and apple vinegar.
4. Immersion of specimens in distilled water, in 3.8% sodium perborate, and in 0.5% sodium hypochlorite did not influence the keratinocytes cell metabolism regardless of the storage period and acrylic resin type.

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