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RESEARCH AND EDUCATION

Antimicrobial effect and cytotoxic activity of vinegar-hydrogen peroxide mixture: A possible alternative for denture disinfection

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ABSTRACT

Statement of problem. Soaking dentures in vinegar or hydrogen peroxide does not seem to remove the microorganisms involved with prosthetic stomatitis efficiently. A mixture of these 2 substances may be effective, but studies are lacking.

Purpose. The purpose of this in vitro study was to evaluate the antimicrobial effect and cytotoxic activity of vinegar-hydrogen peroxide mixtures against *Candida albicans* and *Staphylococcus aureus*.

Material and methods. For antimicrobial tests, planktonic cells and biofilms of *C. albicans* and *S. aureus* cultured on acrylic resin disks were exposed to 0.5% sodium hypochlorite; 0.2% peracetic acid; vinegar-hydrogen peroxide mixtures at concentration ratios 1:1, 1:3, and 3:1; vinegar-water mixtures at concentration ratios 1:1, 1:3, and 3:1; and hydrogen peroxide-water mixtures at concentration ratios 1:1, 1:3, and 3:1. Antimicrobial activity was evaluated by counting viable colony-forming units after disinfection. For cytotoxicity tests, the 1:1 vinegar-hydrogen peroxide mixture was serially diluted (10^{-1} to 10^{-4}) and allowed to be in direct contact with HaCaT keratinocytes for 24 hours. Cytotoxicity was quantitatively and qualitatively determined by counting the number of viable cells and analyzing morphological cell changes.

Results. All vinegar-hydrogen peroxide mixtures, sodium hypochlorite, and peracetic acid efficiently eliminated *C. albicans* and *S. aureus* ($P<.05$), whereas vinegar and hydrogen peroxide solutions used separately were not as efficient as the experimental mixtures. The 10^{-3} and 10^{-4} dilutions of vinegar-hydrogen peroxide solutions were considered noncytotoxic, whereas dilutions below 10^{-2} were strongly cytotoxic, comparable with the 10^{-2} dilution of 0.2% peracetic acid.

Conclusions. The vinegar-hydrogen peroxide mixture effectively eliminated *C. albicans* and *S. aureus* from acrylic resin. Dilutions equal or below 10^{-2} of this mixture presented strong cytotoxic effects. (J Prosthet Dent 2019;121:966.e1-e6)

The use of chemical solutions complements the mechanical removal of denture biofilm, being an adjuvant in denture stomatitis treatment, particularly for elderly patients.[1,2] The solutions that have been evaluated for the effective disinfection of complete dentures,[3-10] except for 0.5% and 1% sodium hypochlorite and high-level disinfectants (2% glutaraldehyde and 0.2% peracetic acid),[6,8-10] reduce acrylic resin microbial counts but are not able to eliminate yeasts effectively.[4,5,7,11-16] However, few studies have investigated the interaction of cleaning approaches for acrylic resin disinfection,[17-19] and the authors are unaware of studies investigating the combination of cleansing solutions. Vinegar and hydrogen peroxide have

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Clinical Implications

A vinegar-hydrogen peroxide mixture in a single solution is a possible alternative for denture disinfection because it effectively eliminates *C. albicans* and *S. aureus* from acrylic resin. Nevertheless, the acrylic resin must be carefully rinsed after disinfection because the solution residues may have strong cytotoxic effects on the oral mucosa.

been separately tested for denture disinfection,[4,5,11,14,15] but studies with a mixture of vinegar and hydrogen peroxide in a single solution as an alternative agent for denture disinfection are lacking. In the food industry, the combination of vinegar and hydrogen peroxide has been more effective at reducing bacterial counts than the isolated use of 1% acetic acid or 3% hydrogen peroxide.[20] Low-toxicity alternatives to eliminating microbes effectively with minimum effect on the properties of acrylic resin are of clinical interest.[2,6]

New disinfection substances and their combinations need to be tested for their toxic properties in addition to their antimicrobial effectiveness, particularly the mixture of acetic acid (vinegar) and hydrogen peroxide, which can have a synergistic effect or react to form peracetic acid.[21-25] Problems regarding the residual cytotoxicity of disinfectant solutions arise when food, scaffolds, and medical or dental devices are either poorly rinsed or their surfaces undergo adsorption and sorption of the disinfection liquids. The toxic effects of oxidant agents may include irritation of the mucosa, skin, eyes, and respiratory tract,[25-28] as well as cellular damage.[22,27,29,30] Although studies have investigated the antimicrobial and cytotoxic effects of isolated vinegar, hydrogen peroxide, and peracetic acid,[22,31,32] studies assessing the cytotoxic effects of a vinegar-hydrogen peroxide mixture are lacking.

The purpose of this study was to evaluate the antimicrobial effect of a vinegar-hydrogen peroxide mixture against *Candida albicans* and *Staphylococcus aureus* as well as its cytotoxic activity against keratinocytes in an attempt to develop a low-cost product with household chemicals which could be used by denture wearers for effective denture disinfection. The null hypotheses were that the combination of vinegar and hydrogen peroxide would have no effect and that no difference would be found between the combination and another commercially available disinfection solution.

MATERIAL AND METHODS

For assessing antimicrobial activity in planktonic cultures, *C. albicans* (ATCC 90028) and *S. aureus* (ATCC 29213) were grown from frozen stocks in Sabouraud agar (Merck KGaA) or brain heart infusion (BHI) agar (Merck KGaA), respectively, in plates for 48 hours at 37 °C. Colonies were loop-transferred to 10 mL of BHI broth (Merck KGaA) supplemented with 1% glucose (Vetec Quimica Fina Ltd). After 24-hour incubation at 37 °C, microbial suspensions were centrifuged at 3042 rpm for 10 minutes at room temperature. Supernatants were discarded, the pellets were resuspended in sterile saline solution (NaCl 0.9%), and microbial turbidity was adjusted to 0.5-McFarland scale (Probac do Brasil Produtos Bacteriologicos Ltd), corresponding to 1×10^8 CFU/mL. From these initial suspensions, aliquots of 2 mL were individually transferred to sterile tubes that were centrifuged for 5 minutes under the same conditions described previously.

Pellets of *C. albicans* or *S. aureus* were washed once with sterile saline solution and suspended in 1 mL of the following freshly prepared solutions: sterile distilled water (negative control); 0.5% sodium hypochlorite (SH–positive control) (Asfer; Asfer Industria Quimica Ltd); 0.2% peracetic acid solution (PAA–positive control) (Voxilon AN; Thech Desinfeccao Ltd); 1:1 mixture of white wine vinegar containing 4% acetic acid (Castelo; Castelo Alimentos S/A) and 3% hydrogen peroxide (AAHP1) (Farmax; Distribuidora Amaral Ltd Farmax); 1:3 mixture of white wine vinegar and 3% hydrogen peroxide (AAHP2); 3:1 mixture of white wine vinegar and 3% hydrogen peroxide (AAHP3); 1:1 dilution of vinegar and distilled water (AA1); 1:3 dilution of vinegar and distilled water (AA2); 3:1 dilution of vinegar and distilled water (AA3); 1:1 dilution of hydrogen peroxide and distilled water (HP1); 3:1 dilution of hydrogen peroxide and distilled water (HP2); and 1:3 dilution of hydrogen peroxide and distilled water (HP3).

After exposure to the disinfection solutions, each microtube was vortexed (AP-56; Phoenix) for 1 minute, the microbial suspensions were serially diluted (10^{-1} to 10^{-5}), and the aliquots were distributed in Saboraud agar plates that were incubated for 24 hours at 37 °C. Viable colony-forming units (CFU) were counted under a stereomicroscope, and the results were expressed as logCFU/mL. Experiments were carried out in triplicate.

The second experiment involved antimicrobial activity in biofilms of *C. albicans* and *S. aureus* formed on acrylic resin disks. For this, sample size calculation indicated a minimum of 10 acrylic resin disks per group to have 90% power at 5% significance.[7] Therefore, 180 heat-polymerized acrylic resin disks (30-mm diameter and 5-mm height) (Onda Cryl; Artigos Odontologicos Classico Ltd) were processed in a microwave oven (Electrolux; MEF41) according to the manufacturer's instructions. The disks were machine polished (APL-4; Arotec) for 2 minutes with a sequence of 320-, 400-, 600-, and 1200-grit abrasive papers, followed by 1-minute brush application of pumice slurry, and 1 minute of 20-μm calcium carbonate universal paste (Asfer; Asfer Industria Quimica

Ltd) on a flannel wheel. After polishing, the specimens were kept in distilled water for 12 hours at room temperature to eliminate residual monomer. Surface roughness was measured (SE1700 Surfcorder; Kosaka Laboratory Ltd) at 0.01-mm resolution, 0.8-mm length, 2.4-mm percussion, and 0.5-mm/s speed. The acrylic resin disks with a mean surface roughness of between 0.05 and 0.10 μm were included. The specimens were randomly distributed into 8 groups and sterilized with hydrogen peroxide gas (Sterrad 100NX; Johnson and Johnson Medical Devices Co).

Stimulated saliva was collected from a 24-year-old nonsmoking volunteer with good oral and systemic health (institutional ethical committee approval protocol #714.626, CAAE 32209614.0.0000.5347) and processed as previously described.[33] Polished acrylic resin disks were inserted in 24-well plates (EP-51-25243; EasyPath) and covered with 1 mL of processed saliva for 45 minutes under continuous stirring (Kline CT-150; Cientec) to allow salivary pellicle formation. The saliva was discarded, and 1.5 mL of BHI broth supplemented with 1% glucose was inserted into each well. A total of 0.5 mL of *C. albicans* or *S. aureus* inoculum were pipetted inside the wells and incubated for 24 hours at 37 °C, after which the culture medium was replaced with fresh BHI broth supplemented with glucose. The plates were then incubated for an additional 24 hours at 37 °C. After 48 hours of biofilm formation, the resin disks were immersed into 2 mL of each disinfection solution for 10 minutes as follows: sterile distilled water (negative control); 0.5% sodium hypochlorite solution (SH–positive control); 0.2% peracetic acid solution (PAA–positive control); 1:1 mixture of white wine vinegar and 3% hydrogen peroxide (AAHP1); 1:3 mixture of white wine vinegar and 3% hydrogen peroxide (AAHP2); 3:1 mixture of white wine vinegar and 3% hydrogen peroxide (AAHP3); 1:1 vinegar-water solution (AA); and 1:1 mixture of 3% hydrogen peroxide and water (HP). The disks were removed from the disinfection solutions and transferred to microtubes containing sterile saline. The tubes were vortexed, and the suspensions were serially diluted (10^{-1} to 10^{-7}) and plated in Saboraud agar plates that were incubated for 24 hours at 37 °C. Viable colony-forming units (CFUs) were counted under a stereomicroscope, and the results were expressed as logCFU/mL. Microbial viable cell counts from both experiments were compared by using 1-way ANOVA and the post hoc Tukey test (α=.05).

For the cytotoxicity experiments, the international standard ANSI/AAMI/ISO 10993-5:2009/(R) 2014 was followed.[34] A total of 5 mL of the 1:1 mixture of vinegar and hydrogen peroxide (AAHP1) was freshly prepared. The solution was also diluted in Dulbecco's Modified Eagle's (DMEM) medium (GIBCO; Thermo Fisher Scientific Inc) at the following serial dilutions: 10^{-1} to 10^{-4}.

Table 1. Mean ±standard deviation for *C. albicans* and *S. aureus* counts (logCFU/mL) in planktonic cultures after treatment with different disinfection solutions

Groups (n=3)	*C. albicans*	*S. aureus*
Water (negative control)	6.85 ±0.04^{b}	7.6 ±0.30^{B}
SH	0^{a}	0^{A}
PAA	0^{a}	0^{A}
AAHP1	0^{a}	0^{A}
AAHP2	0^{a}	0^{A}
AAHP3	0^{a}	0^{A}
AA1	6.69 ±0.05^{b}	6.17 ±0.63^{B}
AA2	6.78 ±0.04^{b}	6.72 ±0.08^{B}
AA3	6.75 ±0.04^{b}	6.22 ±0.03^{B}
HP1	6.22 ±0.13^{b}	4.43 ±0.72^{C}
HP2	6.48 ±0.13^{b}	6.77 ±0.08^{B}
HP3	5.09 ±0.04^{b}	4.29 ±0.52^{C}
P	<.001	<.001

AA1, 1:1 in volume vinegar-water mixture; AA2, 1:3 in volume vinegar-water mixture; AA3, 3:1 in volume vinegar-water mixture; AAHP1, 1:1 in volume vinegar-hydrogen peroxide mixture; AAHP2, 1:3 in volume vinegar-hydrogen peroxide mixture; AAHP3, 3:1 in volume vinegar-hydrogen peroxide mixture; HP1, 1:1 in volume hydrogen peroxide-water mixture; HP2, 1:3 in volume hydrogen peroxide-water mixture; HP3, 3:1 in volume hydrogen peroxide-water mixture; PAA, 0.2% peracetic acid; SH, 0.5% sodium hypochlorite. Different lowercase letters indicate significant statistical difference ($P\leq.05$) in pairwise comparisons of *C. albicans* groups, with ANOVA and post hoc Tukey tests.Different uppercase letters indicate significant statistical difference ($P\leq.05$) in pairwise comparisons of *S. aureus* groups, with ANOVA and post hoc Tukey tests.

Untreated cells cultured for 24 hours under the same conditions with DMEM medium as well as cells cultured with commercial 0.2% PAA solution without DMEM medium (2000 ppm of peracetic acid) were used as negative and positive controls, respectively.

The human keratinocyte cell line HaCaT (#T002000; AddexBio) was cultured in 10-cm culture plates with DMEM-high glucose culture medium supplemented with 10% fetal bovine serum (GIBCO; Thermo Fisher Scientific Inc) and 1% penicillin-streptomycin (GIBCO; Thermo Fisher Scientific Inc), at 37 °C, 95% relative humidity, and 5% CO_2. After reaching 80% confluence, the cells were washed once with ×1 phosphate-buffered saline (PBS) (GIBCO; Thermo Fisher Scientific Inc), incubated with 0.02% ethylenediaminetetraacetic acid (EDTA) (Labsynth Laboratory Products Ltd) for 10 minutes, trypsinized with 0.1% trypsin-EDTA solution (GIBCO; Thermo Fisher Scientific Inc), and seeded at 3×10^{5} cells/mL density in each well of a 12-well plate. After 24 hours, the cells reached approximately 80% confluency, and the medium was discarded. The disinfection solutions were added to the wells in triplicate and allowed to come into direct contact with the cells for 24 hours.

For cytotoxicity quantitative analysis, cells were trypsinized, and the floating cells were stained with Trypan Blue dye (GIBCO; Thermo Fisher Scientific Inc). The total number of viable cells was counted under ×10 inverted light microscope (Axio Observer.Z1; Carl Zeiss Microscopy). The following classification was used[34]:

Table 2. Mean ±standard deviation for *C. albicans* and *S. aureus* counts (logCFU/mL) in biofilm cultures formed on acrylic resin disks after treatment with different disinfection solutions

Groups (n=10)	*C. albicans*	*S. aureus*
Water (negative control)	7.09 ±0.85[b]	5.81 ±1.72[B]
SH	0[a]	0[A]
PAA	0[a]	0[A]
AAHP1	0[a]	0[A]
AAHP2	0[a]	0[A]
AAHP3	0[a]	0[A]
AA1	5.97 ±1.85[b]	5.52 ±2.29[B]
HP1	4.97 ±1.48[b]	0[A]
P	<.001	<.001

AA1, 1:1 in volume vinegar-water mixture; AAHP1, 1:1 in volume vinegar-hydrogen peroxide mixture; AAHP2, 1:3 in volume vinegar-hydrogen peroxide mixture; AAHP3, 3:1 in volume vinegar-hydrogen peroxide mixture; HP1, 1:1 in volume hydrogen peroxide-water mixture; PAA, 0.2% peracetic acid; SH, 0.5% sodium hypochlorite. Different lowercase letters indicate significant statistical difference ($P \leq .05$) in pairwise comparisons of *C. albicans* groups, with ANOVA and post hoc Tukey tests. Different uppercase letters indicate significant statistical difference ($P \leq .05$) in pairwise comparisons of *S. aureus* groups, with ANOVA and post hoc Tukey tests.

percentages of cell viability above 80% are noncytotoxic; within 80% to 60%, weakly cytotoxic; 60% to 40%, moderately cytotoxic; and below 40%, strongly cytotoxic. A plating efficiency over 70% of the control group was considered noncytotoxic. Qualitative changes in general morphology, vacuolization, detachment, cell lysis, cell growth, and membrane integrity were assessed and classified as follows[34]: none, slight, mild, moderate, or severe cytotoxicity. Dilutions of the tested mixture were analyzed using linear regression, and the percentages and morphology of viable keratinocytes were compared descriptively.

RESULTS

For both *C. albicans* and *S. aureus* under planktonic and biofilm conditions, the use of AAHP1, AAHP2, or AAHP3 completely inhibited microbial growth in a similar way as PAA and SH solutions ($P>.05$) (Tables 1 and 2). The microorganism counts exposed to these solutions were lower than the counts found in the presence of the isolated solutions (AA or HP) ($P<.05$). The groups HP1 and HP3 reduced *S. aureus* counts in planktonic cultures ($P \leq .05$) but did not eliminate them (Table 1). For *S. aureus* biofilm growth, HP1 was similar to all AAHP groups and inhibited microbial viability ($P=1.000$) (Table 2).

The cytotoxicity tests showed that the 1:1 AAHP1 solution was strongly toxic, with less than 40% viable cells, whereas highly diluted AAHP1 mixtures were less cytotoxic, promoting a greater cell survival rate (R=0.87) (Fig. 1). The cell reduction rate was 33.43% for the 10^{-3} serial dilution, which is considered noncytotoxic (Table 3). Dilutions bellow 10^{-2} had less than 19.56% cell rate reduction compared with the untreated control group and were considered strongly cytotoxic (Table 3).

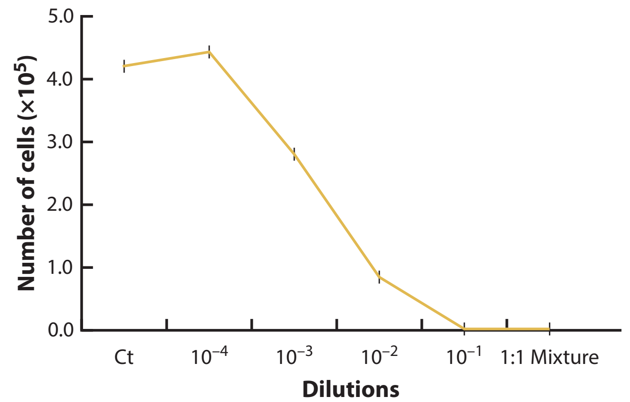

Figure 1. Average number of viable keratinocytes after 24-hour exposure to negative control (Ct), vinegar-hydrogen peroxide mixture (AAHP), and its serial dilutions.

Table 3. Percentages of keratinocyte survival compared with negative control group (regular media) after treatment with several dilutions of vinegar-hydrogen peroxide mixture (AAHP) and 0.2% peracetic acid (PAA)

Groups(n=3)	Percentages of Cell Survival (%)
Negative control	100.0
10^{-4} dilution AAHP	100.0
10^{-3} dilution AAHP	66.57
10^{-2} dilution AAHP	**19.56**
10^{-1} dilution AAHP	**0**
1:1 mixture AAHP	**0**
0.2% PAA	0

AAHP, vinegar-hydrogen peroxide mixture; PAA, peracetic acid. Dilutions that induced less than 40% viable cells are indicated in bold.

The qualitative findings showed that cells treated with the 10^{-3} dilution followed the same morphological patterns of untreated cells, whereas dilutions equal or below 10^{-2} were moderately to severely cytotoxic and had more than 50% floating cells, disrupted membranes, and dark granules inside the cytoplasm, similar to the 0.2% peracetic acid solution (Fig. 2).

DISCUSSION

The present findings rejected the first null hypothesis and supported the second. The AAHP mixtures (AAHP1, AAHP2, AAHP3) were highly effective against the tested microorganisms and presented the same antimicrobial activity and cytotoxicity as the commercially available peracetic acid and sodium hypochlorite solutions. Furthermore, the combination of vinegar and hydrogen peroxide was better able to eliminate these 2 microorganisms from acrylic resin than the separate solutions of vinegar or hydrogen peroxide. Bell et al[20] also reported the effectiveness of a mixture of 1% acetic acid and 3% hydrogen peroxide for the disinfection of beef specimens that had been inoculated with *E. coli*, *L. innocua*, and *S. wentworth*. The protocol used in previous studies

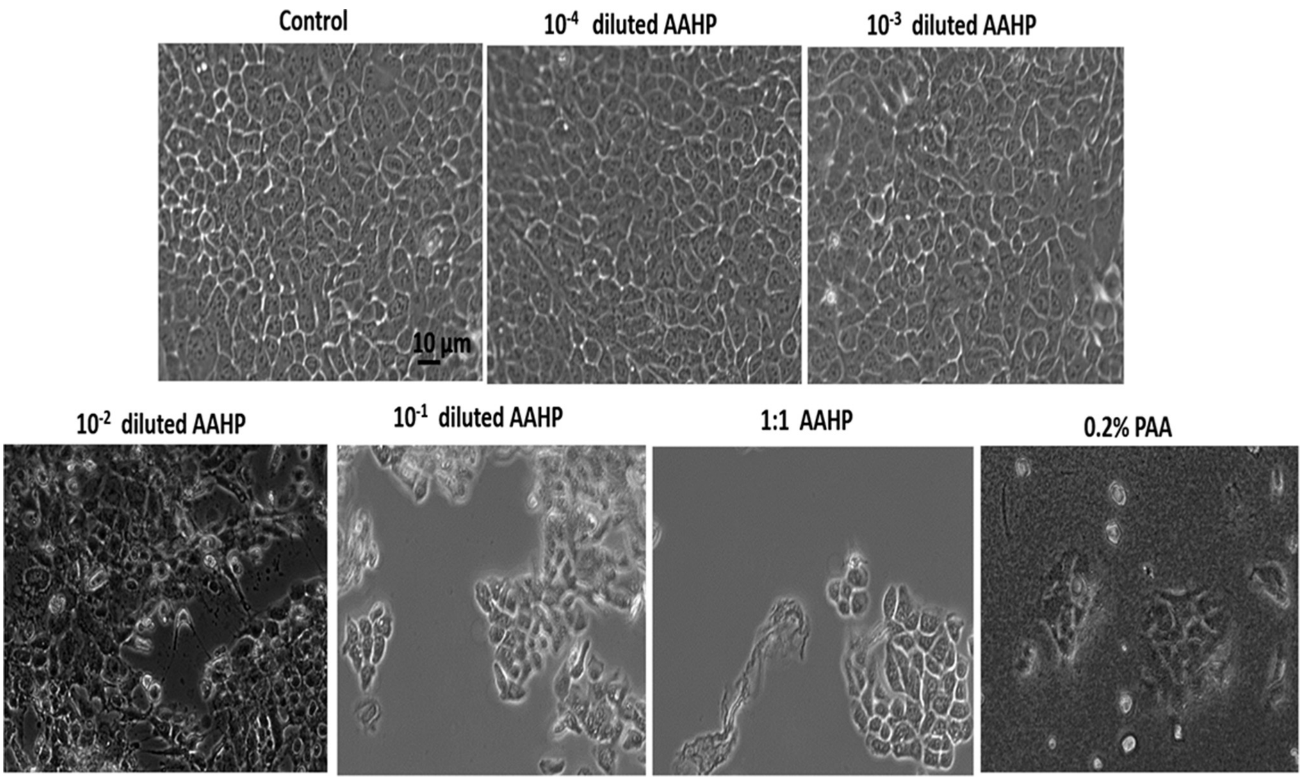

Figure 2. Keratinocyte morphological changes after 24-hour exposure to negative control, vinegar-hydrogen peroxide mixture (AAHP), and its serial dilutions or 0.2% peracetic acid (PAA). Analyzed by photomicrography (original magnification ×10).

included the application of 2 sprays of the solution with a 90-second interval between the applications, whereas the present protocol included immersion in the AAHP solution for 10 minutes.[20] Thus, this mixture seems a promising alternative, not only for food disinfection but also for dental applications, particularly denture disinfection against stomatitis-related microbes.

When mixed together, acetic acid and hydrogen peroxide potentially have a synergistic effect or even react to form peracetic acid.[21-25] The combination of acetic acid and hydrogen peroxide may be analogous to a peroxyacetic acid sanitizer effect in that the antimicrobial effect of this mixture may be related to the reduced pH caused by the synergistic effect of organic acid in combination with hydrogen peroxide; the greater the acid, the greater the microbial reductions.[25-28]

The present study demonstrated that when either vinegar or hydrogen peroxide solution was used separately, *C. albicans* counts were reduced, but not effectively eliminated from the acrylic resin biofilms. Concentrations above 50% in volume of hydrogen peroxide solution had antimicrobial efficacy against *S. aureus*, but not against yeasts. These results corroborate previous findings.[2-5,7,11-16] The present study used a standardized 10-minute disinfection time which may have been insufficient for the antimicrobial action of single solutions. Similar results were found when dentures were immersed in either a 10% red wine vinegar solution during the night or in 100% white wine vinegar for 10 minutes per day for 10 days[5,15] Other studies concluded that the contact time of the vinegar with the denture was an important requirement for its antimicrobial outcomes. These studies reported that apple cider and alcohol vinegars with 4% acetic acid had fungistatic actions after 30 minutes and fungicidal actions after 120 minutes of yeast exposure.[11,14]

The present study demonstrated that increasing concentrations of AAHP were increasingly toxic to keratinocytes, reducing the number of adherent viable cells. It has been reported that pure hydrogen peroxide solution is toxic to keratinocytes and induces oxidative stress responses.[32] Besides the accumulation of cytoplasmic granules, other studies of keratinocytes have described the accumulation of mitochondria-derived reactive oxygen species and the disruption of cytoskeletal filaments in response to harmful substances, which may affect the cell respiratory processes.[30] Solutions with 30 ppm of peracetic acid have been reported to be cytotoxic to HeLa cells, whereas a 3-ppm solution was not toxic.[22] In addition, the solution of 1000 ppm of peracetic acid was considered toxic to L929 fibroblasts.[31] The present study evaluated keratinocyte responses, and the authors are unaware of a previous study investigating the residual cytotoxicity of a vinegar-hydrogen peroxide mixture. Thus, when outcomes are compared, the study results may vary according to different cell characteristics.

CONCLUSIONS

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

1. The mixture of vinegar and hydrogen peroxide can effectively eliminate *C. albicans* and *S. aureus* from acrylic resin.
2. Dilutions equal or below 10^{-2} of this mixture present strong cytotoxic effects, with significant cell death and morphological changes.

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