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In vivo biocompatibility of an interim denture resilient liner containing antifungal drugs

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ABSTRACT

Statement of problem. Antifungal agents incorporated into interim denture resilient liners have been suggested as an adjunct treatment for denture stomatitis (DS). However, before applying this protocol to humans, biocompatibility analysis of such drugs in animal models is required.

Purpose. The purpose of this animal study was to evaluate the in vivo biocompatibility of an interim resilient liner modified with minimum inhibitory concentrations (MICs) of antifungal drugs for *Candida albicans* biofilm.

Material and methods. Sixty Wistar rats were divided into 6 groups (n=5): PC=positive control/no protocol; IOD (intraoral device)=rats using an acrylic resin palatal device (PD); Tru=rats using a PD relined with Trusoft; and Ny (nystatin), Chx (chlorhexidine diacetate), and Ke (ketoconazole) groups=rats using a PD relined with Trusoft + drug MICs. The rats were sacrificed at 7 or 14 days of trial. Histopathological qualitative analysis was performed by comparing photomicrographs of histological sections of the intermolar region. Morphological changes in the epithelium and keratin were quantitatively analyzed by computerized planimetry, and data were analyzed by using 2-way ANOVA and the Tukey HSD test (α=.05).

Results. Quantitative analysis showed that only PD containing Ke significantly decreased the thickness and area of the keratin compared with the other groups (P<.001), which showed no differences between each other (P>.05). These results agreed with those of qualitative analysis.

Conclusions. Incorporation of MICs of Ny and Chx in Trusoft did not induce histopathological changes in the rat palatal mucosa, suggesting the in vivo biocompatibility of this DS treatment. (J Prosthet Dent 2019;121:135-42)

Denture stomatitis (DS) is the most frequent type of oral candidosis and the most common mucosal alteration in the elderly. Its main etiological factor is the colonization of denture-bearing mucosa and acrylic resin bases by *Candida* spp.[1] Treatment of this condition is challenging because it is associated with high rates of clinical relapse and recurrence after conventional therapy with topical and systemic antifungal drugs.[1-7] These therapies require patient compliance because of their strict drug regimen.[8,9] The therapy is further hampered by the unpleasant taste of topical drugs, interaction of systemic drugs with other medications, and need for higher drug concentrations to achieve effective levels, which may exert hepatotoxic and nephrotoxic effects.[10] In this context, the feasibility of using a drug delivery system involving the incorporation of anti-infective drugs in denture base materials has been studied.[8-10]

This system allows the slow and continuous release of drugs into infected tissues and requires only the use of dentures by users, thus eliminating the need for patient compliance to antifungal drug regimens.[8] Moreover, adding drugs to an interim denture resilient liner or

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Clinical Implications

Incorporation of nystatin and chlorhexidine in interim denture resilient liners may be a safe treatment for denture stomatitis because no histopathological changes in the palatal mucosa of rats were observed, suggesting the in vivo biocompatibility of this protocol.

tissue conditioner eliminates the contact between the infected denture and injured tissues, thus preventing reinfection.[11] As a consequence, the denture readapts to the supporting mucosa, resulting in patient comfort during the life cycle of the interim resilient liner,[11] which corresponds to the duration of conventional treatment of DS with topical antifungal drugs (14 days).[8,9] During this period, the use of interim resilient liners modified with drugs also favors the recovery of injured tissues because of the cushioning effect, thus preventing biofilm formation until the denture is replaced or relined with long-term materials.[12]

Although adding antifungal drugs in commercially available concentrations to interim denture resilient liners can effectively inhibit the growth of *C. albicans*,[8,9,13-15] it may affect the structural, physical, and mechanical properties of these liners.[9,16-20] In the search for antifungal concentrations compatible with the properties of these materials, Bueno et al[21] determined the minimum inhibitory concentrations (MICs) of antifungal drugs for inhibiting biofilm formation by *C. albicans* SC5314 in interim resilient liners for up to 14 days. The lowest MICs able to inhibit 90% or more of fungal biofilm were obtained for nystatin (Ny), chlorhexidine diacetate (Chx), and ketoconazole (Ke).[21]

The possible cytotoxic effects of methyl methacrylate, dibutyl phthalate, and other soluble substances released by interim denture resilient liners have been assessed in vitro by using mouse fibroblasts.[22] However, in vitro studies are clinically restricted because they only show direct effects of the released compounds on cells rather than on complex oral tissues and do not replicate the dynamic conditions of the oral environment. Moreover, limited information is available on the in vivo biocompatibility of denture resilient liners with oral tissues.[23,24] To the best of the authors' knowledge, no study has investigated the biocompatibility of drug-modified denture liners with oral tissues. Therefore, the present in vivo study used a rat model and performed qualitative and quantitative histopathological analyses to evaluate the biocompatibility of an interim resilient liner modified with antifungal drugs, which have been recommended for DS treatment, at their MICs for inhibiting biofilm formation by *C. albicans*. The alternative hypothesis was that incorporating antifungal drugs at their MICs would affect the biocompatibility of the interim denture resilient liner during their life cycle (14 days).

MATERIAL AND METHODS

The study evaluated 60 male Wistar rats (*Rattus norvegicus* var. albinus) aged 90 days and weighing 250 to 300 g that were provided by the Central Animal House of Bauru School of Dentistry, University of São Paulo. The experimental protocol was in accordance with the Guiding Principles for the Care and Use of Animals and the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Animal Research of the Bauru School of Dentistry, University of São Paulo (process no. CEEPA 34/2013).

To evaluate the biocompatibility of the interim denture resilient liner, acrylic resin palatal devices (PDs) simulating removable dentures were used. All rats received water ad libitum and were housed in cages in a climate-controlled room, which had 12-hour light-dark cycles, a 23°C ±1°C temperature, and 40% to 80% humidity. They were fed a diet containing a thick paste obtained by grinding the standard pellet diet (Presence; Socil Evialis Nutrição Animal Ind Co Ltd) with tepid water.[23,25] This diet was initiated for the rats 3 days before placement of the PDs, and the animals were monitored daily.[25] At the end of the study, all rats maintained or increased their weight, and neither fur darkening nor prostration due to apparent undernourishment was noted.

For the intraoral procedures, the rats were anesthetized with an intramuscular injection of ketamine hydrochloride (0.4 mL/kg; Dopalen; Sespo Ind Co) and xylazine hydrochloride (0.02 mL/kg; Anasedan; Sespo Ind Co) and then placed on a stabilizing bed.[25]

The PDs were individually fabricated[25,26] with Type IV gypsum (Durone IV; Dentsply Sirona) definitive casts obtained from polyether (Impregum; 3M Brazil) maxillary impressions of each rat and individual autopolymerizing acrylic resin trays. The PDs were waxed to a 3-mm thickness[27] on the respective casts, and the waxed PDs on the casts were embedded in flasks with a dental stone by using a conventional flasking procedure. Subsequently, the PDs were fabricated in a denture base acrylic resin (Lucitone 550; Dentsply Sirona) polymerized by using the short-cycle processing recommended by the manufacturer (90 minutes at 73°C and 30 minutes at 100°C). Excess resin was removed with a mini-cut bur (NB364SE; Dhpro - Rhadartrade Co Imp Peças Ltda - ME), and the cameo surfaces of the intraoral devices were polished with a felt disk and polishing paste (Fotoacrill PFA01; Dhpro - Rhadartrade Co Imp Peças Ltda - ME). The molar region of the PDs was relieved with bur (no. 2; Microdont Ind Co Ltda) to create space for cementation.[25] Before placement,

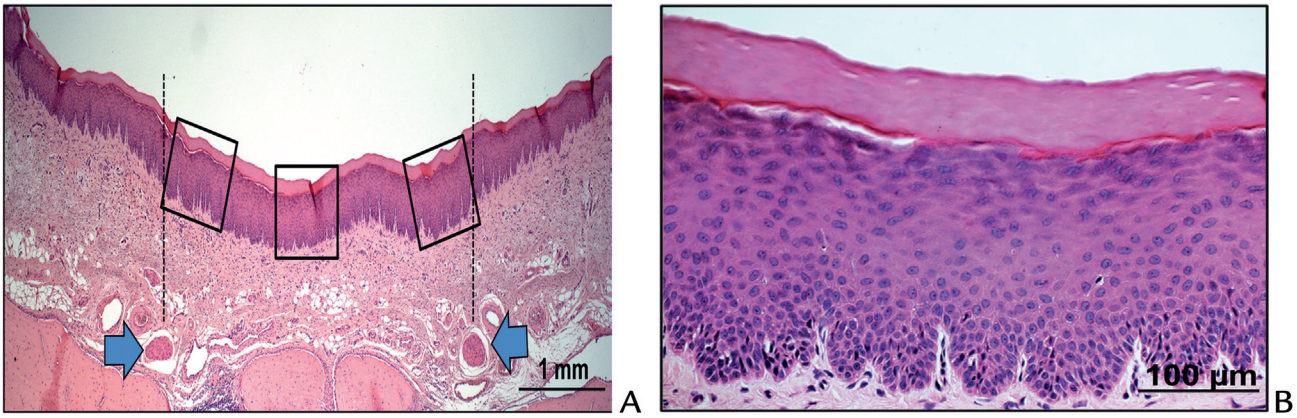

Figure 1. Photomicrograph of histological sections selected for analysis. A, Photomicrograph of transverse histological section of palate in area between first molars; rectangular areas correspond to 3 fields selected for analysis and limited by neurovascular bundles (blue arrows); HE staining, ×10. B, Region of interest selected for analysis; HE staining, ×40. HE, hematoxylin and eosin.

the PDs were brushed with coconut soap and were kept in distilled water at 37°C ±1°C for 48 ±2 hours.[27]

Sixty animals were distributed into 6 groups (n=5)[24] in 2 evaluation periods (7 or 14 days): PC (positive control) rats were fed the standardized paste diet and did not use any PD; IOD (intraoral device) rats using PDs lacking a liner; Tru rats using PDs relined with the interim denture resilient liner (Trusoft; Bosworth Co); and Ny (Pharma Nostra Comercial), Chx (Acros Organics), and Ke (Galena Química e Farmacêutica Ltda) rats, respectively, using a PD relined with Trusoft containing the drug MICs.

A uniform 1-mm space was created in the intaglio palatal surface of the PDs to be relined.[25,27] For the Tru group, Trusoft was prepared following the manufacturer's instructions. In the experimental groups, the drug powders at their MICs for *C. albicans* biofilm (0.032 g for Ny, 0.064 g for Chx, and 0.128 g for Ke per g of resilient material powder)[21] were added to the resilient liner powder, and then the liquid was added to the powder mixture. Next, the resilient liner modified or not with the drugs was inserted in the intaglio palatal region of the PDs. After plasticization (5 to 6 minutes), excess material was removed. To better stabilize the devices in position, autopolymerizing acrylic resin was placed in the relief area of the molars for the cementation of the PDs.[25]

At the end of the 7 or 14 days, the rats were sacrificed by administering an anesthetic overdose. Next, the maxillae were fixed in 10% buffered formalin for 7 days and decalcified for 8 weeks in 4.13% ethylenediaminetetraacetic acid. Then, they were washed, dehydrated, and embedded in paraffin. Transverse semiserial sections of the posterior palate (5-µm thick) were cut at 100-µm intervals and were stained with hematoxylin and eosin (HE).[25] All histological sections were assigned to a numerical sequence to code the experimental conditions.

Qualitative histological analysis was performed by visually comparing images of the mesial to distal surfaces of the first molars limited by the neurovascular bundles (RI) captured from 5 histological sections per animal. A microscope (Axioskop 2; Carl Zeiss AG) equipped with a ×40 immersion objective was used. Quantitative histological analysis was also performed by using 5 sections per animal. For each maxilla, 15 microscopic fields (3 fields per histological section) of the posterior palate comprising the RI were examined (Fig. 1) by using a ×40 immersion objective and digital analysis software (AxioVision 4.8; Carl Zeiss AG).[23] Nine variables were evaluated: lengths of the corneal surface (CSL), of the basal surface (BSL), and the ratio of BSL to CSL; thicknesses of keratin (KT), of cellular compartment (CCT), and the ratio of CCT to KT; and areas of keratin (KA), of the cellular compartment (CCA), and the ratio of CCA to KA. CSL, BSL, KA, and CCA were obtained by averaging the measurements obtained from 15 fields (Fig. 2A and 2C). For KT and CCT, 6 measurements or fields were obtained, as illustrated in Figure 2B (total of 90 measurements), because the thickness varied depending on the assessed region.

The measurements of the examiner (J.H.) had been previously calibrated by an experienced histology technician (T.M.C.). At 20 days after the initial measurements, 15 histological sections from PC, IOD, and Tru groups treated for 7 days were evaluated, and the intra-examiner results were compared. The paired *t* test was used to determine systematic error (α=.05), and the Dahlberg formula was used to estimate casual error. The paired *t* test did not show statistically significant differences between the first and second measurements of the 9 variables (P>.05). The value of casual error also did not exceed the representative values of significant error. Test-retest reliability was confirmed by an intraclass correlation coefficient greater than 0.82 between the measurements.

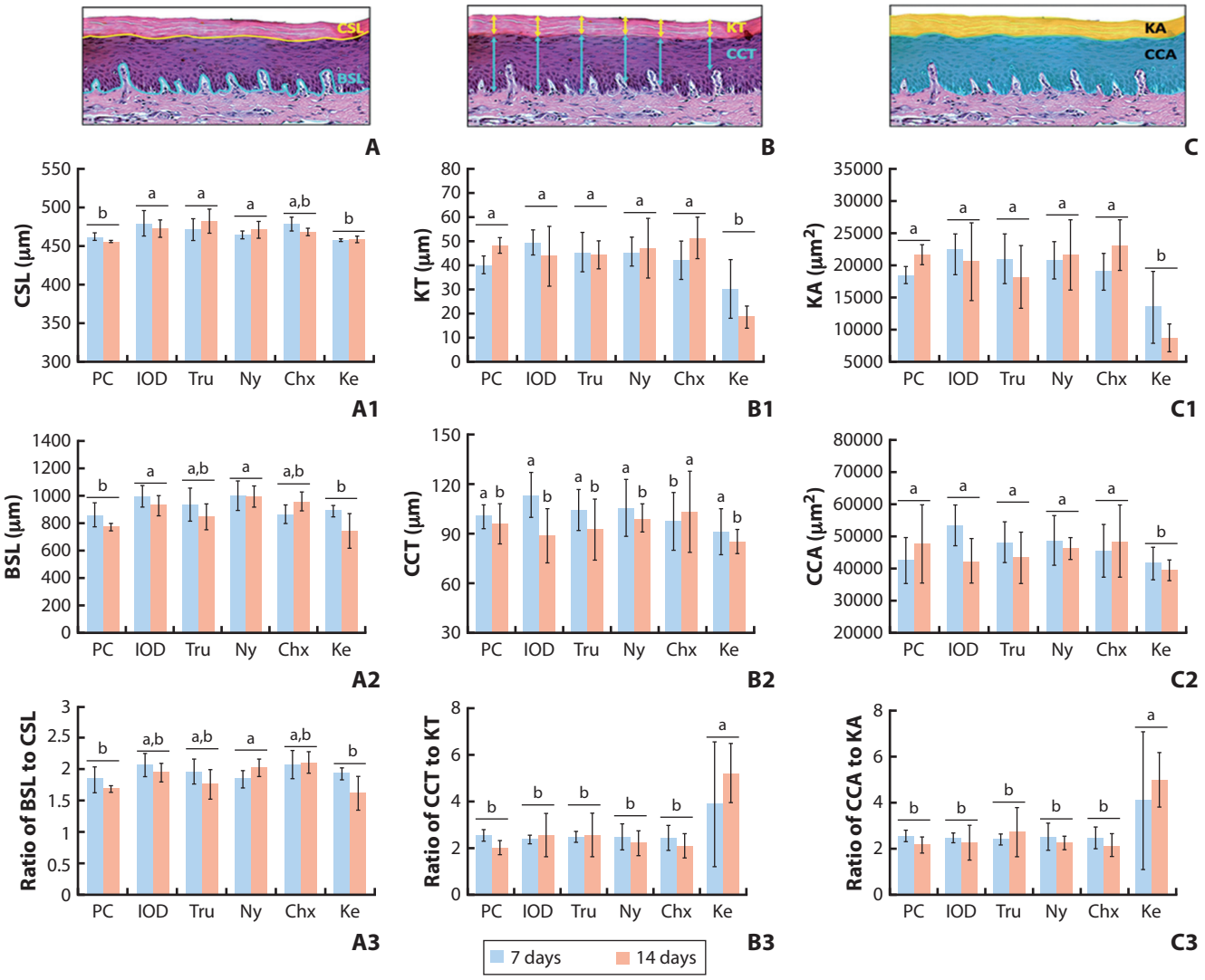

Figure 2. Quantitative histological analysis of study groups at 7 and 14 days. Representative illustration of measurements. A, Length. B, Thickness. C, Area. Groups connected by horizontal bar did not differ significantly ($P>.05$). Groups with different lowercase letters differed significantly ($P<.05$).

Initially, a multiple regression model was used to assess the normality and homogeneity (Shapiro-Wilk test) of data distribution for each variable (SigmaPlot for Windows v12.0; Systat Software Inc). Subsequently, all variables were analyzed by using 2-way ANOVA (for factors 'study group' and 'period') followed by the Tukey HSD post hoc test ($\alpha=.05$).

RESULTS

Qualitative histopathological analysis was performed by evaluating the photomicrographs as seen in Figure 3. The thicknesses of the stratum corneum (yellow line) and of the cellular compartment (blue line) of rats treated for 14 days were lower than those observed in rats treated for 7 days. The basal surface in the Ke group showed less pronounced rete ridges (black arrow) compared with those in the other groups at 14 days.

The Shapiro-Wilk normality test showed P values higher than 0.05. ANOVA only detected a significant difference for the independent variable 'group' in the dependent variables CSL, KT, KA, BSL, CCA, and all ratios (BSL to CSL, CCT to KT, and CCA to KA) ($P<.001$). CCT analysis only showed a significant difference for the independent variable 'period' ($P=.047$). For CSL (Fig. 2A1), PC and Ke groups showed the lowest values, while IOD, Tru, and Ny showed the highest values ($P<.05$), with no significant difference from Chx. For BSL (Fig. 2A2), PC and Ke groups showed the lowest values, while IOD and Ny showed the highest values ($P<.05$), with no significant difference from Tru and Chx. For the ratio of BSL to CSL (Fig. 2A3), PC and Ke groups showed lower values than Ny group ($P=.001$), with no significant difference from IOD, Tru, and Chx groups. Regarding KT (Fig. 2B1), the Ke group showed the lowest values and therefore the highest ratio of CCT to KT (Fig. 2B3)

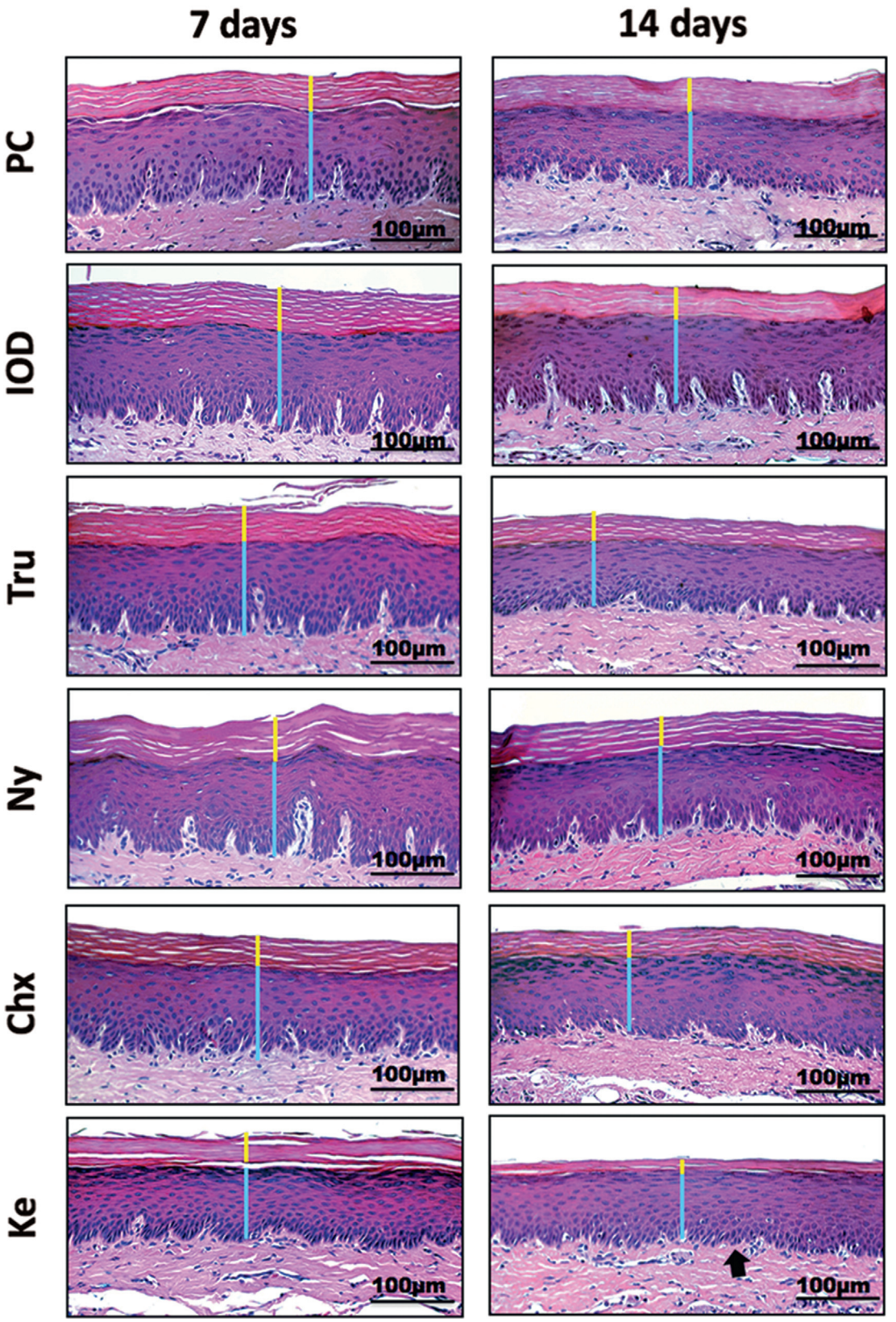

Figure 3. Photomicrographs of epithelial region of rats in different study groups at 7 and 14 days. Hematoxylin and eosin staining, ×40.

regardless of evaluation period ($P<.001$), and no significant difference was found among the other groups. For CCT (Fig. 2B2), all experimental groups exhibited lower values after 14 days of treatment than after 7 days, except the Chx group, which demonstrated higher values after 14 days than after 7 days of treatment ($P=.047$). In the analysis of area (Fig. 2C), the Ke group had the lowest values regarding KA (Fig. 2C1) and CCA (Fig. 2C2) and consequently the highest ratio between them (Fig. 2C3) ($P<.001$), and no difference was found among the other groups.

DISCUSSION

The alternative research hypothesis that incorporating antifungal drugs affected the biocompatibility of the interim resilient liner during their life cycle was partially accepted. Quantitative histopathological analysis of the oral tissues of rats wearing PDs compared with those of rats in the PC group at 7 and 14 days showed that Trusoft containing Ny and Chx at MICs did not significantly affect the mean measurements of the variables analyzed. However, thickness of keratin and area of keratin and cellular compartment were lower in rats of Ke group than those observed in the other groups. These results were also noted in the qualitative analysis.

In the current study, as PDs were designed to completely adapt to the palatal mucosa, they were properly relined and remained stable throughout the life cycle of the interim resilient liner. To meet these requirements, the following parameters were defined: paste diet[23,25] and individually fabricated PDs[25,26] retained on the molars by cementation.[24,25] The 7-day period was selected because this is the average time during which palatal mucosa may show inflammatory infiltrates.[25] The 14-day period corresponds to the duration of conventional DS therapy with topical antifungal drugs and to the life cycle of interim resilient liners.[8,9,22] These changes in the methodology also helped determine the criteria used for histopathological analyses: obtaining histological sections of palatal soft and hard tissues that transversely comprised the first molars, from one neurovascular bundle to the other.[23,25]

Qualitative and quantitative histopathological analyses also showed lower thickness of the cellular compartment at 14 days in comparison with that observed at 7 days in rats wearing or not wearing (PC group) PDs. Previous studies[23,24] did not show these changes at 14 days in rats wearing PDs relined with a soft material without drugs, which may be attributed to the differences in methodology used, such as the design of PDs and processing of histological specimens.

Ny is considered the first-line agent for topical treatment of uncomplicated cases of oral candidiasis in patients with normal immune function,[4] with the advantages of few side effects, low cost, and no reported drug interactions.[6] Besides the rare detection of fungal strains resistant to Ny,[16] this polyene antifungal antibiotic is poorly absorbed by the intestinal tract when in suspension, so most is excreted without undergoing any change, thus reducing hepatotoxicity.[3] The Ny dose recommended for adults is 400 000 to 600 000 U 4 times a day. When applied intraperitoneally, the median lethal dose (LD_{50}) of Ny in mice is 200 mg/kg. The analyses performed in this study suggested the biocompatibility of the Ny-containing interim resilient liner for up to 14 days because no histopathological changes were observed in the palatal mucosa of rats compared with those in rats in the PC group. This may be attributed to the use of the lowest MIC of Ny,[20] which was gradually released in the oral cavity.[8,20] Results of simultaneous research (unpublished results) and those of the MIC determination[21] corroborate these findings, demonstrating, by UV-visible spectrophotometry, the sustained release of Ny at MICs from Trusoft for up to 14 days in water immersion. Thus, the use of an Ny-containing interim resilient liner may be a promising DS treatment, because this infection is primarily caused by *Candida* spp, which are sensitive to this drug.

Cationic detergent Chx, the best wide-spectrum bisguanidine antimicrobial drug, is widely used in antiseptic and denture disinfectant solutions to prevent and treat oral infections. Chx ruptures the cell membrane of *C. albicans* and other yeasts present in the prosthetic biofilm, which promotes the development and maintenance of DS. Moreover, the occurrence of Chx-resistant species has not been reported to date.[16] Also, Chx shows significant substantivity and hence is effective for longer periods, especially when used in a powdered form.[9,10,14,16,21] Gradual Chx release directly at the infection site[14,15] as well as controlled topical release[10] may help overcome the secondary systemic effects or drug-drug interactions of Chx, thus assuring its availability in the infected area at therapeutic doses for a long period. In addition, during the MIC's determination, Bueno et al[21] also observed with UV-visible spectrophotometry the sustained release of Chx at its MIC from Trusoft for 14 days (unpublished results). In this period, 26 mg of Chx was released from the material. LD_{50} of Chx in mice when administered orally is 2 g/kg. Therefore, Chx released from the interim denture resilient liner at the concentration tested in the present study may be an excellent option for treating DS because of its biocompatibility with denture-supporting tissues.

A biofilm formed on a prosthesis is not a random mixture of microorganisms but is an organized structure that varies over time according to the metabolic properties of cells and local conditions. Therefore, wide-spectrum antimicrobial drugs are more appropriate than antifungal drugs for inhibiting fungal and bacterial

growth in biofilms formed on prostheses. Moreover, the gradual release of Chx at MIC from an interim denture resilient liner decreases the possibility of allergic reactions in the host because its released concentration is within the nontoxic range.[14] However, the present study focused on the possible local effects of Trusoft, modified or not by antifungal drugs, on tissues supporting intraoral devices rather than on its systemic effects.

Azole antifungal drugs such as Ke are fungistatic; however, they may also have fungicidal effects.[5] The initial daily oral dose of Ke is 200 mg; this dose may be increased up to 400 mg daily in patients with more severe or resistant infections. In contrast, the LD_{50} of Ke in mice is 166 mg/kg when applied intraperitoneally. Ke is associated with advantages such as prompt absorption (systemic) after oral administration and effective action against fungi, including *Candida* species. However, the U.S. Food and Drug Administration warns that Nizoral (Ke oral tablets, 200 mg) can cause severe liver injuries and adrenal gland problems, or drug interactions.

However, even if all Ny or Ke incorporated in Trusoft was released within 1 day, which is not expected,[10,14] the released concentration would still be within the daily recommended dose of these drugs. This demonstrates that the Ny or Ke concentration added to the interim denture resilient liner in the present study was much lower than the concentration that induces acute toxicity when administered all at once. Moreover, the effects of Ke on the thickness of the stratum corneum suggested that it did not induce systemic toxicity. Despite that, Ke should be carefully used even when administered using the drug delivery system tested because keratin reduction may increase the susceptibility of the oral mucosa to infections. Beyond the potential tissue injury effect of Ke, histological findings for this drug may also be due to the administration of this drug at higher MIC than that of the other drugs. The Ke dose was 4 times higher than that of Ny and 2 times higher than that of Chx in the current study.

One limitation of this study was that only 1 brand of interim resilient denture liner was evaluated. In addition, because biocompatibility is a complex property, future studies with similar methodology should be conducted by using other important analyses, including molecular analysis. Also, the in vivo evaluation proposed with the acrylic resin devices tested in this study presents limitations in that important factors such as removal of the tested intraoral devices and their hygiene and deformation because of occlusal load during normal feeding conditions could not be evaluated. Thus, the clinical performance of the modified resilient material and its biocompatibility should be evaluated by performing clinical studies involving individuals wearing removable dentures.

CONCLUSIONS

Within the limitations of this in vivo study, the following conclusions were drawn:

1. Incorporating ketoconazole at MICs to the biofilm of *C. albicans* in the interim denture resilient liner significantly decreased the thickness and area of the stratum corneum of the palatal mucosa of rats ($P<.05$).
2. The addition of nystatin and chlorhexidine at MICs to the interim denture resilient liner resulted in no significant changes in the mean measurements of the histopathological variables analyzed ($P>.05$) in the palatal mucosa of rats.

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