Retentive efficacy, antimicrobial and cytotoxicity comparisons between different types of commercial and experimental denture adhesives with antifungal action

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The effect of the addition of nystatin and an alternative antifungal derived from pyrazoles in different commercial denture adhesives on their retentive efficacy, cytotoxicity and antimicrobial activity against Candida albicans was evaluated. Commercial denture adhesives were prepared with the inclusion of nystatin and 3,5-diaryl-4,5-dihydro-1H-pyrazole-1-carboximidamide (pyrazole) in three concentrations: 23.78 %w/w, 3.02 %w/w, and 0.31 %w/w (0.015 g, 0.0015 g, and 0.00015 g, respectively). The retentive efficacy was tested observing the influence of the medium, type of commercial denture type and the test condition (dipping). The antifungal action through disk diffusion and direct contact tests at 1, 4, 8 and 12 h and cytotoxic activity was evaluated in mouse fibroblasts (NIH/3T3) by the MTT reduction colorimetric assay. The addition of pyrazole and nystatin in commercial denture adhesives did not affect retentive efficacy rates and enhanced antifungal actions against Candida albicans. Results show a possibility of using denture adhesives as a delivery system for commercial antifungals (Nystatin) or pyrazole, with the second concentration (1,560 µg–3.02 %w/w) as the most efficient.

Keywords: Antifungal agents, Denture adhesives, Disk diffusion antimicrobial tests, Pyrazoles, Toxicity tests

INTRODUCTION

The use of conventional complete dentures is still very usual for the oral rehabilitation of complete edentates¹. This rehabilitation device is still quite prevalent in underdeveloped and developing countries. One of the main factors for the successful rehabilitation with complete dentures is their stability and retention². This fact does not always depend on dentists and the clinical steps for the manufacture of oral prostheses³,⁴. Consequently, since they have been widely commercialized and commercially disseminated, denture adhesives would be a resource to increase the prostheses’ retention and stability⁵.

Since the end of the 18th century, denture adhesives have been used to improve the retention of dentures, and the first scientific report on denture adhesives occurred in 1935 by the Council of Dental Materials of the American Dental Association⁶. It is known that 30% of total denture wearers use or have used denture adhesives, and that the number of denture adhesive users varies between 15 and 33% of prosthesis users. Within a year, 55 million units of prosthetic adhesives were sold in the United States, totaling more than 220 million dollars⁷.

Denture adhesives feature several commercial types, such as powder, tape, and cream, whilst their action is due to the absorption of liquids from the medium, which enhances viscosity and retentive efficacy⁸,⁹. In spite of their functional and psychological advantages for total oral prostheses patients, several disadvantages may be pointed out when these types of denture adhesives are employed. They include maladaptations¹³, bone resorption¹³, increase in the vertical dimension¹⁴, accumulation of biofilm and others. The accumulation of fungal biofilm raises discussions on the possibility of these adhesives predisposing wearers to diseases common to users of oral prostheses, such as chronic atrophic candidiasis¹⁵-¹⁸.

Chronic atrophic candidiasis is a fungal infection that affects up to 67% of dental prostheses wearers¹⁹. The pathology is mainly caused by Candida albicans, but other Candida spp. may be associated²⁰. It is actually a multifactorial disease with the involvement of factors that exceed only the existence and presence of C. albicans²¹,²². However, the accumulation of fungal biofilm in a favorable, humid, rough, and dark environment, greatly increases the chance of establishing

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this infection.

The treatment of chronic atrophic oral candidiasis is broad and includes the use of topical antifungal agents, replacement of prostheses and oral hygiene. Two major drug groups stand out: polyeneic and azole derivatives. As polyeneic derivatives, nystatin and amphotericin-B may be highlighted. In current study, nystatin has been selected as an antifungal material because the drug has now been used in antifungal therapy for more than fifty years. In addition, nystatin is one of three main polyene drugs used in the treatment of mycoses. The parenteral administration of nystatin is followed by severe side effects, but oral and topical administration of this antifungal agent is not related to any significant toxic side effects. Consequently, the antifungal agent is currently used in superficial candidiasis treatment, such as oral and vaginal infections with topical or oral administration only. Azole derivatives maybe divided into two groups: imidazoles and triazoles. They have basically the same mechanism of action, namely, inhibiting the synthesis of ergosterol in pathogenic fungi, increasing the permeability of the cytoplasmic membrane and, consequently, the loss of intracellular constituents. There is not only a noticeable increase in the occurrence of cases of oral candidiasis, but evidence has been detected on the increasing resistance of the C. albicans against antifungal agents currently commercialized, which makes mandatory the discovery of new drug alternatives. These alternatives must be more effective, less toxic and cheaper. One alternative comprises the new pyrazoline compounds with activities against the microorganisms, low cytotoxicity and high yield, allowing their production on a large scale. Heterocyclic compounds containing pyrazole have a broad spectrum of biological activities, such as monoamine oxidase inhibitor, anticonvulsant, antibacterial, hypnotensive, antipyretic and anti-inflammatory activities. The pyrazole nucleus is an aromatic azole heterocycle with two adjacent nitrogen atoms. Pyrazole derivatives have exhibited a broad spectrum of biological activities. Approved pyrazole-containing drugs include celecoxib, antipyrine, phenylbutazone, rimonabant, and dipyrone. Several research groups have synthesized and evaluated pyrazoles against different biological agents. Since 3,5-diaryl-4,5-dihydro-1H-pyrazole-1-carboximidamides have shown antimicrobial capacity and low cytotoxicity, they are an alternative source for the treatment of fungal infections caused by Candida.

Current studies test the retentive efficacy and antimicrobial activity against C. albicans, and the cytotoxicity of different commercial types of denture adhesives containing either nystatin or an alternative antifungal agent derived from pyrazole.

### MATERIALS AND METHODS

#### Pyrazoles synthesis

The synthesis and initial tests of antifungal activity are described by Oliveira et al., wherefrom the initial antifungal concentration of pyrazole to be tested has been established.

#### Denture adhesives types and compositions

For current tests, only one commercial brand (Corega, GlaxoSmithKline, Brentford, UK) has been used in the three commercial types of denture adhesive: powder, tape and cream. The compositions of the products are described in Table 1. Commercial denture adhesives were prepared with the inclusion of nystatin (N) and (Pyrazole —P) at three concentrations: 23.78 %w/w, 3.02 %w/w, and 0.31 %w/w (0.015 g, 0.0015 g, and 0.00015 g, respectively). The incorporation of the antifungal agent (pyrazole or nystatin) in the tape was done by impregnation with the compound. Inhibition incorporated the antifungal agent in the powder and cream. Table 2 provides the proposed groups and compositions.

#### Characterization of retentive efficacy

Retention was measured in triplicate; twenty tests were replicated and performed in a pair of acrylic resin specimens by universal tester described in a previous study. The acrylic resin cylinders, dimensions of 25×55 mm, were made stable in the universal testing machine. Test was carried out by applying 0.3 g of denture adhesive already formulated to the polished surface of the resin cylinders (denture base). Further, 2 KgF was applied to the acrylic resin cylinder for 15 s to ensure a consistent application force. The weight was removed for 30 s and finally, the sets were separated. The forces required to pull the resin cylinders were measured and recorded. The adhesion force measurements were made on a universal testing machine, operating with a 100 N load cell at a speed of 1 mm/min. The physical adhesive strength between acrylic resin and denture adhesive was measured between the denture adhesive

### Table 1  Composition of the different types of Corega® (Glaxosmithkline, Brentford, UK) commercial denture adhesives

| Commercial type | Composition                                      |
|-----------------|--------------------------------------------------|
| Powder          | Partial Sodium Salt —Poly Calcium (Methylvinylether/Maleic Acid 49.80%; Carboxymethylcellulose 49.80%; Mint flavor |
| Cream           | Sodium/Calcium poly salts (Methylvinylether/Maleic Acid), carboxymethylcellulose, mineral oil and Vaseline |
| Tape            | Sodium carboxymethylcellulose, microcrystalline wax, polybutylene and polyethylene glycol |
and the denture base, and the wettability of the denture adhesive to artificial saliva during the dipping as ‘the denture retentive efficacy’.

The ‘dipping or immersion’ test comprised weighing of denture adhesive and its placement on the polished surface of one of the specimens, previously wetted with 0.5 mL of artificial saliva. This amount of liquid was measured with a previously calibrated automatic pipette. Test comprised the set of specimens (resin cylinders) plus denture adhesives immersed in a device coupled to the testing machine, with 20 mL of artificial saliva so that the whole set was submerged. Tensile bond strength was registered in MPa.

Antimicrobial assays
1. Agar diffusion test
Methodology by Sassone et al. was used as a parameter to perform the agar diffusion test. C. albicans strain (ATCC 62342) was acquired from the Microbiology Laboratory of the Faculty of Dentistry at Federal University of Pelotas. These strains were cultured prior to the test in a Mueller-Hinton broth (Becton Dickinson Company, Sparks, MD, USA) at 37°C, for 48 h. After cultivation, a collection and retrieval with sterile swabs was made for test tubes containing phosphate-saline buffer (PBS). These tubes were calibrated on the Mcfarland 0.5 scale. Mueller-Hinton broth plates (Becton Dickinson Company) were inoculated and the material was placed on the plate approximately 15 mm from the other material. Inhibition zone was measured from the circumference of the disk to the margin where microorganism growth occurred.

Further, classification proposed by Karaman et al. was used, where: sensitive products are those that promote inhibition zone ≥3 mm or ≥than positive control; moderately sensitive products are those that promote inhibition zone ≥2 mm, but less than positive control; resistant products are those that promote inhibition zone ≤2 mm. Positive control was the denture adhesive without the addition of antifungal agent, whilst negative control was the adhesive with nystatin.

2. Direct contact test
Direct contact test was conducted according to Damlar et al. Strains selected were those used for the agar diffusion test. Consequently, 24 h before the test, the strains were picked in BHI agar medium and incubated at 37°C. The inoculum was prepared by dissolving an aliquot of the microorganism in BHI broth medium (Becton Dickinson Company) following the 0.5 MacFarland scale (1×10^6 colony forming units). From this inoculum, 100 µL in each well of a 96-well microtiter plate were retrieved and the denture adhesives, manipulated according to Table 2, were added. The plates were incubated for 1, 4, 8 and 12 h. Afterwards, 240 µL BHI broth was added in each well; the plates were placed for 5 min in a plate shaker and 100 µL of each well were added to 900 µL of BHI broth (Becton Dickinson Company) and serial 1:10 dilutions were performed in 4 aliquots. Further, 25 µL of the dilution were seeded in Petri dishes containing Mueller-Hinton broth (Becton Dickinson Company) and incubated at 37°C for 48 h. After this period, two previously trained examiners calibrated the counted colonies. Positive (inoculum without any other product) and negative (culture medium only) controls were administered within each group. Inhibition percentages of each tested denture adhesive were calculated by positive control of each group accepted as 100% growth.

Cytotoxicity assay
1. Cell culture
Current assay used the mouse fibroblast cell line (NIH/3T3) as an experimental model. The cell line maintenance procedures were performed in a laminar flow hood, following protocols for maintaining the sterility of the materials, supplements and culture media used. The cells were kept in cell culture bottles with Dulbecco’s Modified Eagle’s Medium (DMEM) and bovine fetal serum (BFS) in a CO2 oven at 37°C. Cell growth was monitored daily using an inverted phase contrast microscope. Culture medium was changed every 2 or 3 days, according to cell metabolism.

Cell counting
Cells were washed twice in PBS and suspended from the bottom of the flask using 0.25% trypsin solution in PBS and 1% EDTA to determine their number. The contents of each bottle were removed, placed in a test tube containing 5 mL of DMEM and centrifuged, to inhibit the action of

Table 2 Denture adhesives formulation and composition (%w/w) of the tested groups

| Denture adhesive | Nystatin | Pyrazole |
|------------------|----------|----------|
|                  | N1       | N2       | N3 |
| D1               | 23.78    | 3.02     | 0.31 |
| D2               | 23.78    | 3.02     | 0.31 |
| D3               | 23.78    | 3.02     | 0.31 |

§Concentration in %w/w of nystatin or pyrazole in each group material
The sequential numerical indication 1, 2 and 3 in each material indicate different formulated concentrations.
trypsin. The supernatant from the tubes was discarded and precipitates suspended in 1 mL of DMEM. Further, 20 µL of cell suspension were dispensed in a test tube with an additional 20 µL of 0.4% Trypan blue. Outside the laminar flow, a drop of this mixture was placed in a Neubauer chamber (or hematocytometer) and taken to the inverted phase microscope for cell countings.

**MTT assay**

As shown in Table 2, denture adhesives were formulated and placed in DMEM. The cell suspension was plated at a concentration of 2×10⁴ cells per well and distributed in a 96 well cell culture (ELISA) plate. Each well received 200 µL of complete DMEM. The plate was then incubated at 37°C, in 5% CO₂ air, for 24 h. After this period, culture medium was removed from the wells and equal volumes (200 µL) of the experimental material were added to each well. In control wells, 200 µL of DMEM were added. After removing the test extracts, 200 µL of PBS and 20 µL of MTT (tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were added to each well, incubated at 37°C, in the dark, for 24 h. MTT was then aspirated and 200 µL of dimethyl sulfoxide (DMSO) were added to each well. Data from cytotoxicity (absorbance at 570 nm) were determined with a spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA) and submitted to statistical analysis. Eight replicates were performed for each group, and the experiment was repeated three times.

**Statistical analysis**

Statistical comparisons were performed for the immersion (dipping) test with SigmaPlot 12.0 software (Systat Software, San Jose, CA USA). Tensile bond strength between groups was compared by Kruskal Wallis test at \( p < 0.05 \). Statistical comparisons between groups was performed to analyse the agar diffusion test, by Kruskal Wallis and One-way ANOVA at \( p < 0.05 \).

In the case of direct contact test, statistical comparison between groups was performed by Kruskal Wallis and by One-way ANOVA at \( p < 0.05 \). Data from cytotoxicity (absorbance at 570 nm) were submitted to two-way analysis of variance to evaluate independent variables (materials and concentrations) and interactions, followed by pairwise comparisons (Sidak adjustment) and Tukey’s test. The level of significance was set at \( \alpha = 0.05 \). Analyses were performed with SPSS 23 (Statistical Package for Social Sciences, v. 23).

**RESULTS**

There was no statistical difference between the three commercial types (Fig. 1). Consequently, the inclusion of antifungal agents did not impair the retention efficacy of the denture adhesives (\( p > 0.05 \)).

Table 3 shows the results of the disk diffusion test.

![Fig. 1 Comparison of different types of denture adhesives with regard to retentive efficacy (Tensile Bond Strength), at dipping condition.](image-url)

| Groups | Denture adhesive |
|--------|------------------|
|        | Powder | Cream | Tape  |
| Pirazoles |        |        |       |
| 0.015 g | 4*     | 3*     | 5*    |
| 0.0015 g| 3*     | 1*     | 4*    |
| 0.00015 g| 2*   | 1*     | 0*    |
| Nystatin |        |        |       |
| 0.015 g | 4*     | 3*     | 4*    |
| 0.0015 g| 3*     | 3*     | 3*    |
| 0.00015 g| 0*   | 2*     | 0*    |

Median rates (mm) followed by the same superscript letters at each group material in columns indicate no significant differences (\( p \geq 0.05 \)).
According to data obtained, it may be observed, when nystatin and the experimental pyrazole were compared, they promoted an inhibition halo and behaved similarly ($p > 0.05$).

Figure 2 shows antifungal behavior (inhibition %) of different types (powder, cream and tape) of commercial and experimental denture adhesives at different times. In Fig. 2, results are measured as a percentage of inhibition.

Figure 3 shows cytotoxicity results from different types (powder, cream and tape) of commercial and experimental denture adhesives. Regardless of the commercial types (powder and cream), the cytotoxicity of denture adhesives revealed very similar trends (Figs. 3A and B). However, when the material was tested on tape (Fig. 3C), nystatin was not cytotoxic.

**DISCUSSION**

Adhesives for complete dentures have been used to optimize the retention of complete dentures by increasing the adhesive and cohesive properties of saliva and the viscosity of the medium between the prosthesis...
Microbial colonization, especially of and survival of microorganisms and biofilm formation. are seen as an environment highlighting proliferation atrophic candidiasis 18,39). Some studies have observed induce a chronic inflammatory response, called chronic predisposing factor for chronic prosthetic stomatitis15,18). Have pointed out that denture adhesives stimulate the artificial saliva and the dipping method were chosen artificial saliva, using wetting and dipping methods. Result is interesting since the power because the simulation is the closest to what occurs in the oral cavity34). Artificial saliva and the dipping method were tested using distilled water, natural and test method34). Commercial and experimental denture adhesives were tested using distilled water, natural and artificial saliva, using wetting and dipping methods. Results varied according to different means and methods. Artificial saliva and the dipping method were chosen because the simulation is the closest to what occurs in the oral cavity34). Result is interesting since the power of adhesion was tested with the inclusion of antifungal agents regardless of the commercial type.

In a literature review, Grasso6) mentions that prosthetic adhesives may be divided into insoluble and soluble, which vary in their composition, the former includes the tape and the latter group includes cream, paste and powder. The soluble group has synthetic agents which depend on the chemical properties of one or more active ingredients that increase their volume from 50% to 150% and become viscous and sticky in the presence of water or saliva, filling the spaces between the prosthesis base and the supporting tissues. The active ingredients are a mixture of polymer salts with different degrees of solubility in water, which are designed to produce short and long-term adhesives. Carboxymethylcellulose (CMC) and polyvinylether methyl cellulose (PVM-MA) are examples of short- and long-acting salts, respectively. CMC salts provide strong initial retention. Due to their high degree of solubility, they dissolve quickly, losing their efficacy within a short period. PVM-MA salts, however, have low solubility that takes longer to activate, albeit with a longer action period. Later, calcium and zinc salts were added to its composition to improve efficacy.

Even though the tape composition varies between commercial brands, all of them essentially include a manufactured blade impregnated with a water-based active component. Examples of adhesive ingredients include sodium alginate or ethylene oxide polymer, which become sticky when activated with saliva. Difference comprises blade thickness39.

The effect of denture adhesives on the oral microbiota is an interesting aspect since conventional full dentures are seen as an environment highlighting proliferation and survival of microorganisms and biofilm formation. Microbial colonization, especially of Candida spp., can induce a chronic inflammatory response, called chronic atrophic candidiasis18,20). Some studies have observed that the use of prosthetic adhesives has not significantly altered the oral microbiota30,41). However, other studies have pointed out that denture adhesives stimulate the proliferation of Candida spp. and that this could be a predisposing factor for chronic prosthetic stomatitis15,18). Regardless of the adhesive used, the wearer must be aware that these products must be completely removed from the prosthesis and mucosa, as they may harbor microorganisms harmful to oral health. Therefore, care with total prosthesis and oral tissues needs special attention and patients should be well-informed about it30.

The disk diffusion test verified the antifungal efficiency of commercial and experimental fasteners. Some authors point out that this test is a reliable alternative to the microdilution method in broth, reference NCCLS M27-A243). When nystatin is compared with experimental pyrazole, both promoted an inhibition halo and behaved similarly (p>0.05). Result confirms and opens up a potential use for pyrazole as an alternative antifungal compound. The use of pyrazole has already been discussed in another study and its antimicrobial activity had been proven against different species of C. albicans. Low cytotoxicity has been detected31). Among antifungal agents, polyenes (Nystatin and Amphotericin B) and azoles (itraconazole, miconazole and clotrimazole)44,45) are the therapeutic agents employed most for the topical treatment of oral candidiasis. Although widely used, they have certain limitations due to their side effects, such as toxicity and the emergence of resistant strains. The above enhances the need for the development of other compounds with low cytotoxicity and the potential for treatment of mycoses46).

In general, commercial types of commercial denture adhesives were not decisive in the activity of the antifungal agents nystatin and pyrazole (p>0.005). However, in the use of pyrazole as an antifungal agent, an improvement was detected in the action against C. albicans species when the commercial type was on tape (p=0.031), superior to types cream and powder. This may be due to the insoluble characteristics of the tape, as opposed to the soluble characteristics of cream and powder. Thus, due to this insolubility, it is possible to have a greater availability of this active principle and thus a greater antifungal action. Some studies address the antifungal action of commercial denture adhesives without the inclusion of antifungal agents, but they fail to compare the action of the different commercial types and their influence. In current study, denture adhesives did not contain antifungal agents, they did not have the production of an inhibition halo and the consequent inhibitory action on the growth of Candida spp. Another in vitro study observed that, among ten commercial brands, only two induced an inhibitory effect on the growth of C. albicans, but most products induced changes in the macro and microscopic morphology of yeasts and colonies of the fungus47.

In case of the direct contact test, results were similar to those presented by disk diffusion, and the tested pyrazole had results on antifungal activity similar to nystatin (p>0.05). Results were observed for 12 h, or rather, the time period based on the commercial instruction of the product duration of action. All results reaffirm the possibility of using pyrazoles as an alternative to antifungal agents and the possibility of denture adhesive agents as a possible means of delivering antifungal agents.

Cytotoxicity (Fig. 3) was also evaluated, since antifungal activity alone would not be enough for the
positive characterization of the material. The aim of a 1993 study by Ekskrand et al., was to evaluate the cytotoxic effects, or rather, microbial contamination in 19 commercial brands of prosthetic adhesives. All prosthetic adhesives evaluated were cytotoxic and some were more susceptible to microbial contamination, which proved to be more pronounced in adhesives based on “natural” raw materials. In general, current results showed that nystatin and pyrazole were similar at the two lowest concentrations. However, pyrazole was more toxic than nystatin at the highest concentration evaluated. In fact, pyrazole at the highest concentration was the most toxic product among all tested materials evaluated. In fact, pyrazole at the highest concentration was the most toxic product among all tested materials and concentrations [p<0.05 (Fig. 3)]. Regardless of the commercial type (powder and cream), the cytotoxicity of denture adhesives showed very similar trends (Figs. 3A and B). However, when the material was tested as a tape (Fig. 3C), nystatin proved not to be cytotoxic.

CONCLUSION

Based on the methodologies employed and within the limitations of current study, one may conclude that denture adhesives could be used as delivery systems for commercial antifungal agents and that pyrazoles enhanced antifungal activities against Candida albicans. The inclusion of antifungal agents did not affect the adhesion of the denture adhesives. In addition, pyrazole at 1,560 µg (3.02 %w/w) was the most efficient, with the lowest cytotoxic concentration, regardless of the different commercial types of denture adhesives tested.

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