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Poly (Methyl Methacrylate) absorption and releasing of nystatin and fluconazole

Absorção e liberação de nistatina e fluconazol por polimetilmetacrilato

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Abstract

Objectives: The aim of this study was to verify if there are poly (methyl methacrylate) (PMMA) absorption and releasing of nystatin (NYS) and fluconazole (FLZ) after simulated treatment of oral candidosis. **Materials and methods**: Specimens (30 × 25 × 5 mm) prepared with PMMA polymerized by hot water bath or microwave energy were immersed into NYS (3.12 μg/mL), FLZ (2.56 μg/mL) or deionized water (control) during 14 days at 35 ± 2 °C. After treatment simulation, specimens were immersed into distilled water during 3, 7, 10 and 14 days. The immersion liquid was changed after each analysis. Higher performance liquid chromatography was used in order to detect antifungal compounds. In order to determine if there was surface deposition of drugs on PMMA resin, specimens were analyzed with electronic microscopy (SEM). **Results**: None of the antifungal agents was released from the PMMA resins. **Conclusion**: Within the limitations of this study, it could be concluded that PMMA resins had no drug absorption with posterior release.

Keywords: Dental prosthesis. Antifungal agents. Acrylic resins. Chromatography. High pressure liquid.

Resumo

Objetivos*: O objetivo deste estudo foi verificar se o poli (metil metacrilato) (PMMA) é capaz de absorver e liberar nistatina (NYS) e fluconazol (FLZ) após simular um tratamento para candidose oral.* ***Materiais e métodos****: Espécimes (30 × 25 × 5 mm) foram preparados em resina de PMMA por banho de água quente ou energia de micro-ondas e, em seguida, imersos em solução contendo NYS (3.12 µg/mL), FLZ (2.56 µg/mL) ou água deionizada (controle) durante 14 dias a 35 ± 2 °C. Após a simulação de tratamento, os espécimes foram imersos em água destilada durante 3, 7, 10 e 14 dias. O líquido de imersão foi trocado após cada análise. Cromatografia líquida de alta performance foi utilizada para detectar a presença dos agentes antifúngicos. Para determinar se houve deposição dos agentes antifúngicos na superfície de PMMA, os espécimes foram analisados por microscopia eletrônica de varredura (MEV).* ***Resultados****: Não houve liberação de agentes antifúngicos dos espécimes.* ***Conclusão****: Considerando as limitações deste estudo, pode-se concluir que a resina de PMMA não absorve ou libera agentes antifúngicos.*

Palavras-chave*: Prótese dental. Agentes antifúngicos. Resina acrílica. Cromatografia líquida de alta performance.*

Introduction

Denture-related stomatitis is a common intraoral disease, which is associated with high levels of *Candida* adhesion and biofilm formation to a poly (methyl methacrylate) (PMMA) denture base (1). To colonize the oral environment, *Candida* species has the ability to adhere to prosthetic devices and form biofilms consisting of a mixture of yeast and hyphae surrounded by extracellular polymers (2).

Although PMMA resin surfaces could represent an important predisposing factor for microorganisms colonization (3, 4), characteristics as being fracture resistant, satisfactory optical properties, esthetics, easy manipulation, reasonable cost and being inert to oral and other tissues make PMMA resin an elective material for denture fabrication (5-7). However, a drawback of PMMA resins is that polymers used in these materials can absorb water and chemical compounds from the oral environment, and also release components into the surrounding environment (6, 8), which lead to a variety of chemical and physical processes that may result in deleterious effects on the structure and function of dentures (7).

In this context, during denture-related stomatitis therapy, which is commonly treated with nystatin (NYS) or fluconazole (FLZ) (9, 10), drugs compounds could interact with PMMA resin (11-13). Due to the polymeric nature, it is possible that continuous exposure of PMMA resin surfaces to antifungal agents permits their absorption and posterior releasing after treatment interruption, which could be detected as a residual effect. Besides, constant exposure of antifungal compounds at sub-inhibitory concentrations has effects on expression of virulence factors by *Candida* species, increasing extracellular proteinase specific activity and, consequently, biofilm pathogenicity (14).

Therefore, the purpose of this study was to verify if there are PMMA absorption and releasing of nystatin (NYS) and fluconazole (FLZ) after simulated treatment of oral candidosis.

Materials and methods

Experimental design

Specimens measuring 30 × 25 × 5 mm were prepared according to the manufacturer's directions using a water bath-cured or microwave-cured PMMA resin. The specimen surfaces were finished and polished, and surface roughness (SR) measurements were performed for standard purpose. Next, specimens were randomly assigned into three groups according to the treatment employed. Specimens were individually immersed in plastic tubes containing NYS, FLZ or deionized water (control). Immersion solutions were changed daily until completed fourteen days. After, specimens were washed, dried, and then, immersed in plastic tubes containing distilled water. In the 3rd, 7th, 10th and 14th days of immersion, storage solutions were analyzed by high performance liquid chromatography (HPLC) for drugs detection. Data analysis was performed considering three factors: PMMA, immersion period and anti-

fungal agent. Response variable was the amount of NYS or FLZ released and detected by HPLC.

Preparation of PMMA specimens

A hot water bath-cured (Clássico Artigos Odontológicos Ltda, São Paulo, SP, Brazil) and a microwave-cured (Onda-Cryl, Clássico Artigos Odontológicos Ltda, São Paulo, SP, Brazil) PMMA denture bases were used in this study. A wax matrix (30 × 25 × 5 mm) was flasked in Type III dental stone (Herodent Soli-Rock, Vigodent, Rio de Janeiro, Brazil) using a metal dental flask (Uraby; DLC, São Paulo, Brazil) for water bath polymerization or plastic dental flasks (BMF1, Clássico Artigos Odontológicos Ltda, São Paulo, SP, Brazil) for microwave oven. After the dental stone set, wax matrix was eliminated with boiling water. Then, PMMA resins were mixed in accordance with the manufacturer's recommendation and inserted into the flasks at dough stage.

Microwave-cured resin specimens were polymerized in a microwave oven (Continental AW-42, with 2.450 Ghz frequency and 900 W maximum potency; Bosch, Manaus, AM, Brazil) for three minutes at 360 W, four minutes at 0 W and three minutes at 810 W. Specimens polymerized by hot water bath were processed in an automatic polymerization unit (Termotron P-100, Termotron, Piracicaba, SP, Brazil) at 74 °C for nine hours. Once processed, all flasks were allowed to bench cooling for at least three hours and the specimens were removed.

Specimens were ground using progressively smoother aluminum oxide papers (grit 320, 400 and 600) in a horizontal polisher (Arotec APL-4, São Paulo, SP, Brazil). For mechanical polishing, a brush disc with pumice slurry and a felt cone with chalk powder were used. They were also ultrasound cleansed (Thornton T 740, Thornton-Inpec Eletrônica LTDA, Vinhedo, Brazil) for 20 minutes and then immersed in distilled water at 37 °C for 24 hours for residual monomer release (15). After these procedures, specimens were evaluated for SR for standardizing surface properties.

Surface roughness measurement (SR)

For standardized purposes, SR of the acrylic resin specimens was measured using a profilometer (Surfcorder SE 1700, Kozaka Industry, Kozaka, Tokyo, Japan) accurate to 0.01 μm with total measurement length of 3.2 mm and 0.5 mm/s. Three readings were made for each specimen, and a mean value was calculated (12).

Drug preparation

Forty-two specimens of each PMMA resin were randomly assigned to three groups in accordance with the treatment employed: NYS, FLZ or deionized water.

FLU (UK – 049858, kindly donated by Pfizer, Sandwich, UK) and NYS (Sigma-Aldrich Corp., St. Louis, MO, USA) were prepared using deionized water at 2.56 μg/mL (maximum concentration found in human saliva after biotransformation) (16) and 3.12 μg/mL (minimal inhibitory concentration-MIC-for *Candida* spp.) (17), respectively (18). Deionized water was used as a control group for both antifungal agents.

Treatment simulation

Each specimen was immersed in a plastic tube containing 25 mL of prepared NYS, FLU or deionized water. Tubes were protected against light and stored at 35 ± 2 °C in a cabinet for fourteen days to simulate a treatment of oral candidosis. The immersion solutions were changed daily.

After this period, specimens were washed with 15 mL of distilled water, dried and immersed in new plastic tubes containing 25 mL of distilled water. This set was stored at 35 ± 2 °C in a cabinet. After three days, specimens washing and drying procedure were repeated, as well as in the 7^{th}, 10^{th} and 14^{th} days of immersion, when the specimens were discarded. The immersion liquid was changed after each analysis. An aliquot (2 mL) of storage solutions of each period (0, 3^{rd}, 7^{th}, 10^{th} and 14^{th} days) were analyzed by high performance liquid chromatography (HPLC). Each experiment was carried out three times.

High performance liquid chromatography (HPLC)

Chemicals, reagents and standards

Methanol of HPLC-quality, hydrochloric acid and sodium citrate of analytical grade were all purchased

from GCC (England). Standard materials of the drugs, FLU and NYS, were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA).

HPLC apparatus and working conditions

HPLC was performed using a Gilson HPLC system (Gilson, Villiers le Bel, France) equipped with a Gilson 305 pump, a Gilson 805 manometric module, a column heater LC101 (Ecom, Prague, Czech Republic), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) fitted with a 20-µL sample loop and a LC-4B electrochemical detector (BAS, West Lafayette, IN, USA). Peak areas and sample concentrations were calculated with a Gilson 714 chromatography data system.

The HPLC apparatus was operated under the following working conditions: eluent was methanol (50:50)/0.01 M phosphate buffer, followed by injection of 30 µL in a column C-18 (4.6 × 25 cm, particle size 5 µm) at a flow rate of 1 mL/min.

Stock standard solutions and working standard solutions

A 1000 µg/mL stock standard solution was prepared for each antifungal agent by dissolving 10 mg of the drug in 10 mL HPLC-water. These prepared solutions were kept in a refrigerator. The working standard solution for each antifungal agent was prepared by mixing 20 µg/mL of the drug with 50 µg/mL of the internal standard p-methyl phenol in HPLC-water.

Storage solution analyses

Storage solutions analysis was preceded by dissolving in 500 µL of distilled water, 50 µL of stock standard solution of FLU or NYS and 250 µL of 1 M ammonium hydroxide. After shaking, FLU and NYS was extracted with 5 mL of ethyl acetate and centrifuged (5000 g/ 23 °C/ 5 min) to separate the layers. After centrifugation, FLU and NYS were re-extracted by adding 1.5 mL of 6 M ammonium hydroxide and 5 mL of ethyl acetate and, then, new centrifugation. Ethyl acetate was transferred to a clean tube and evaporated at 40 °C on nitrogen gas stream. The residual was dissolved in 200 mL of solvent for column (50:50 methanol/0.01 M phosphate buffer), followed by injection of 30 mL in a column C-18 at a flow rate of 1 ml/min. Representative chromatograms were performed after HPLC analysis in order to verify the presence of antifungal agents in the different storage solutions.

Scanning electronic microscopy (SEM)

In order to determine if there was deposition of drugs rather than absorption by PMMA resin surface after the treatments, specimens were visualized with SEM in high-vacuum mode at 10 kV. SEM was used at x500 or x1000 magnification.

Statistical analysis

All analyses were performed using SAS software (SAS Institute Inc., version 9.0 Cary, NC, USA) employing a significant level fixed at 5%. Study factors were PMMA, immersion period and antifungal agent. Response variable was the amount of NYS or FLZ released and detected by HPLC.

Results

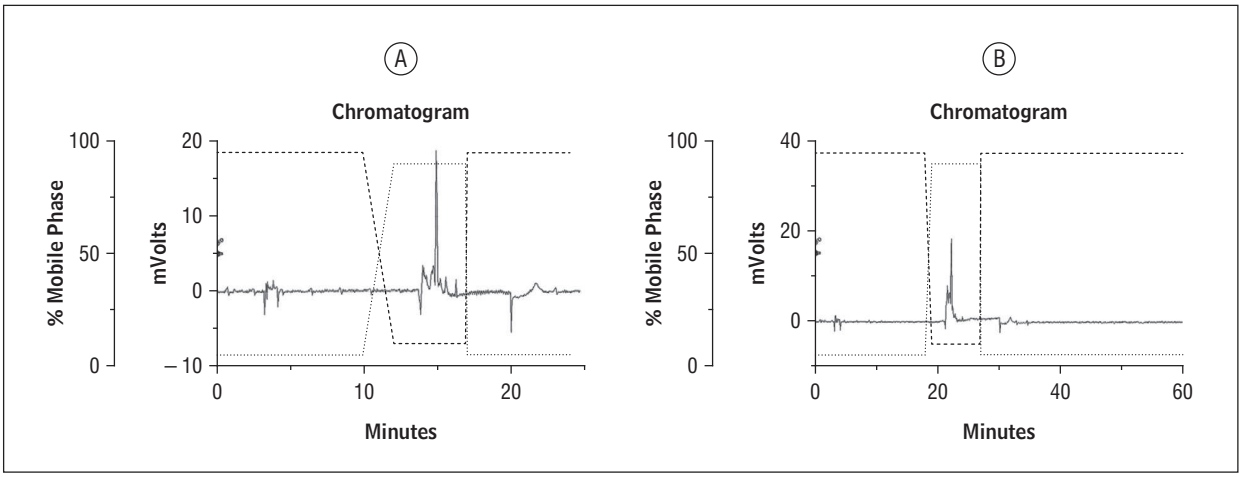
PMMA surface roughnesses were measured for standard purpose. Mean values and standard deviations were 0.08 ± 0.2 for hot water bath-cured resin, and 0.09 ± 0.2 for microwave-cured resin. There was no statistical difference between them.

Chromatography analysis of storage solutions were performed to detected the amount of NYS or FLU released of both PMMA resins at different immersion periods. However, HPLC immersion analyses demonstrated no releasing of NYS or FLZ from both PMMA resins (Graphics 1 and 2).

According to SEM images, both PMMA resins demonstrated no deposition of drugs compounds after the treatments (Figure 1).

Discussion

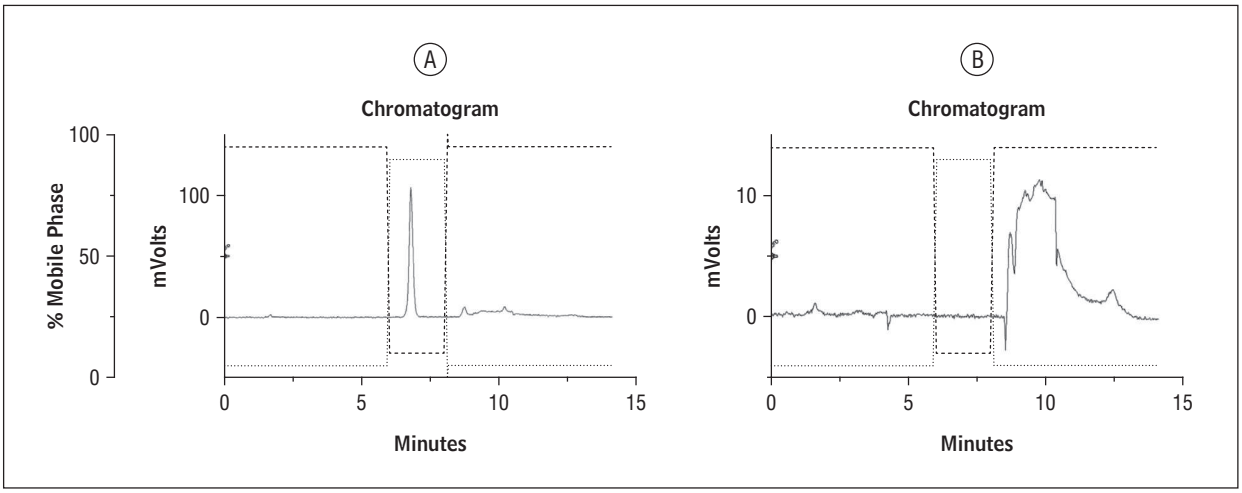
PMMA resin surfaces are important predisposing factor for *Candida* spp. adhesion and theses appliances, with suboptimal hygiene, act as reservoirs



Graphic 1 - Chromatography analysis of NYS

Note: A = representative chromatogram for the stock standard solution of NYS; B = representative chromatogram for the analysis of no releasing of NYS by PMMA specimen after 14 days.

Source: Research data.



Graphic 2 - Chromatography analysis of FLZ

Note: A = representative chromatogram for the stock standard solution of FLZ; B = representative chromatogram for the analysis of no releasing of FLZ releases by PMMA specimen after 14 days.

Source: Research data.

of microorganisms (1, 19). Many authors have highlighted its occurrence on denture bases and its impact on removable denture wearers, such as denture-related stomatitis (2, 9). Regarding the treatment of such disease, the use of FLZ and NYS is well established in literature (9, 10). Despite the availability of the antifungal agents and initial inflammatory condition reduction, clinical aspects are recurrent after treatment suppression (16, 20) and therapy failure is not uncommon (10).

The common recurrence suggests a constant interaction of antifungal agents with the PMMA resin and, consequently, could damage to material surface and eventually favor later colonization by *Candida* species (11-13). However, due to PMMA resin properties of water sorption and release (5-8), it is possible that continuous exposition of such surfaces to antifungal agents permits chemical compounds absorption and, a residual effect could be detected by posterior releasing after treatment interruption.

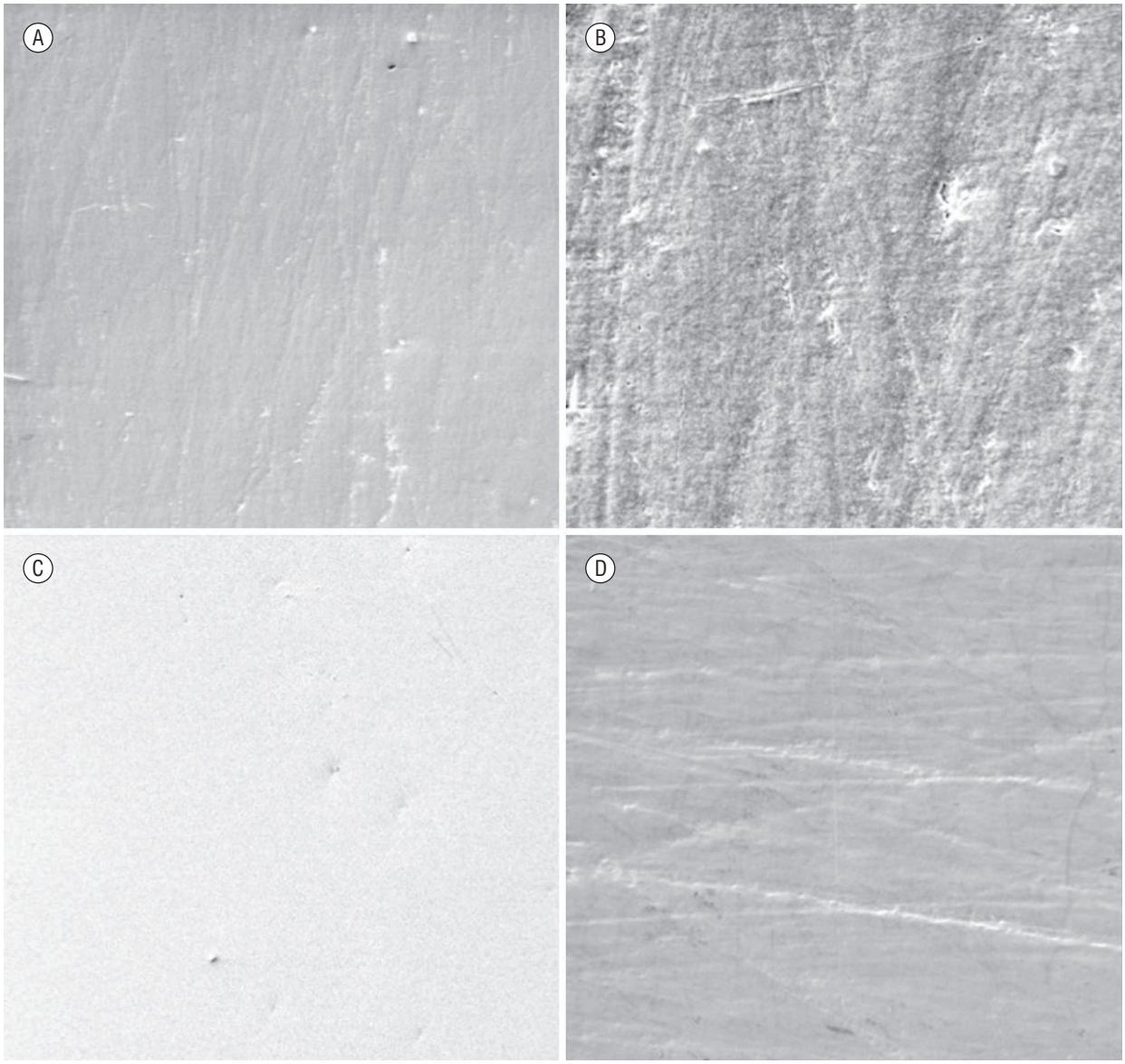


Figure 1 - SEM analyses from hot water bath-cured specimens after 14 days of AA exposure
Note: A = NYS – 100x; B = NYS – 1000x; C = FLU – 100x; D = FLU – 100.
Source: Research data.

This could be critical if the antifungal compounds are released in concentrations below the minimum inhibitory concentration (MIC) for the microorganism, since sub-inhibitory concentrations induces virulence factors secretion and biofilm pathogenicity (14). Thus, safety of PMMA for drugs administration should be investigated.

Although the incorporation of antifungal agents has been previously tested (11, 21, 22), the objective of this study was to simulate the clinical condition of denture-related stomatitis treatment to verify the safety of PMMA resin regarding FLZ and NYS absorption and posterior releasing.

Through the results obtained by HPLC analysis, it was possible to verify that after a treatment simulation of oral candidosis, there are no residual effects from PMMA resin specimens (Figure 1 and 2). Although it has been clearly established that methacrylate-based polymers absorbs up to 30% water depending on the osmolarity of the external solution (23) or the formulation of the particular polymer (11, 22, 23), these results clarifies that antifungal

agents such as FLZ and NYS are not released from PMMA resin matrix. Consequently, this is not a factor that interferes on expression of virulence factors by *Candida* species and biofilm pathogenicity.

The methods of detecting released antifungal drugs varied among investigators. Some used spectrophotometric and agar diffusion measurements of the released drug into water; others employed a proton nuclear magnetic resonance spectroscopy for the same purpose (23) whereas some researchers used ultraviolet spectrometry in the measurement of drug release (24). In the present study the chosen methodology was high liquid performance chromatography (HPLC), which permits a wide range of applications and offers significant advantages in the analysis of pharmaceutical formulations and biologic fluids. An added advantage is that many detectors used in HPLC are non-destructive, thus facilitating sample recovery (11).

Specimens' images obtained by SEM revealed no deposition of drugs after FLZ and NYS treatment (Figure 3). These results are in agreement with Silva et al. (12), who observed that continuous exposition of PMMA resins to chemical components of FLZ and NYS is not able to compromise surface characteristics as surface roughness, surface free energy and surface hardness, and eventually favor later colonization by *Candida* species.

Considering all the advantages that make PMMA resin an elective material for denture bases (5-7), the results here presented suggest that this material is also safe for drugs administrated against denture-related stomatitis.

Conclusions

Within the limitations of this study, regarding a possible absorption and posterior release of drugs administrated against denture-related stomatitis, it is possible to suggest that PMMA resins had no drug absorption with posterior releasing.

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