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Thermopolymerized Acrylic Resin Immersed or Incorporated with Silver Nanoparticle: Microbiological, Cytotoxic and Mechanical Effect

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Associated with the use of removable prostheses, the development of candidiasis, called prosthetic stomatitis, is frequently observed. In view of the application of silver nanoparticles (AgNP) in dentistry that may offer antimicrobial effect, the aim of this study was to evaluate the effect of adding AgNP with different concentrations during thermopolymerization or immersion of acrylic in this substance in the properties antifungal, mechanical and cytotoxic. The groups were divided: addition of 1% silver nanoparticle solution (G1), addition of 2.5% silver nanoparticle solution (G2), addition of 5% silver nanoparticle solution (G3), immersed for 10 min in aqueous silver nanoparticle solution (G4), immersed for 24 hours in aqueous silver nanoparticle solution (G5). In the cytotoxicity assay, at all evaluation times, all groups showed cytotoxic effect ($p < 0.05$) when compared to the control group (CG). For the microbiological assay, *C. albicans* reduction was observed only for G4 and G5 when compared to CG ($p < 0.05$). The lowest resistance values were observed in the group with 5% silver nanoparticle (G3) incorporation ($p < 0.05$). It was concluded that the thermopolymerized acrylic resin immersed in AgNP, G4 and G5 promoted microbiological reduction, cytotoxicity increase and flexural strength decrease at 5% concentration.

Keywords: *stomatitis, silver nanoparticle, Polymethyl Methacrylate.*

1. Introduction

Although implants have achieved relevant prominence in contemporary dentistry[1,2], being the treatment of choice in most cases of multiple dental losses, total removable prostheses still have an important place, especially in situations where implant is contraindicated or when the economic factor is limiting[3].

In patients using a removable complete denture, a strong association with candidiasis, called prosthetic stomatitis, is observed. It consists of a lesion commonly seen under the plaque area of the prosthesis, affecting about 65% of the users of maxillary complete dentures[4]. In addition, saliva influence, biofilm formation and substrate nature, as well as individual and microorganism-related variables, can determine the course of infection[5].

The treatment of choice for prosthetic stomatitis associated with candidiasis is the combination of topical antifungal, patient guidance on prosthesis hygiene (use of brush and solutions), prosthesis polishing, and verification of the need for replacement[6,7]. 2% Miconazole has been successful in its application, to the detriment of other antifungals since it is a[7]. The drug is directly attached to the previously sanitized prosthesis, which acts as a "tray" and gives the drug a longer contact time with the lesion, and consequently better response and faster regression[8]. Nystatin, in turn, a topical antifungal agent widely used for the treatment of other candidiasis subtypes, does not appear to have such a satisfactory effect on the prosthetic stomatitis when compared to miconazole gel, as it is a suspension, which gives shorter time of contact with microorganisms, delaying the desired effect[9].

In this context, highly reactive metal oxide nanoparticles exhibit excellent broad spectrum biocidal action and have been investigated as antimicrobial agents[10,11]. In this sense, silver has been incorporated into various health polymeric materials because it has antimicrobial properties for a broad spectrum of microorganisms, including gram-positive and negative bacteria[12] and fungi[13]. Additionally, the use of silver nanoparticles has promoted greater effect on microorganisms compared to conventional antibiotics[14].

Considering the application of nanoparticles in dentistry, this in vitro study aimed to evaluate whether their addition with different concentrations during thermopolymerization or immersed in the solution has antifungal effect, without influencing the mechanical properties of resins or cytotoxic effect on gingival fibroblasts. In addition, it checks the hypothesis that by increasing the concentration or immersing in silver nanoparticle, antimicrobial power and cytotoxicity and reduced flexural strength would increase.

2. Materials and Methods

2.1 Ethical aspects

As it is an in vitro experimental study (protocol 2017/0739), it was waived by the Research Ethics Committee of Faculdade São Leopoldo Mandic (Campinas/SP-Brasil).

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2.2 Preparation of specimens

The specimens, in thermopolymerized acrylic resin, were made with the aid of metallic matrices of 15 mm diameter x 2 mm thickness for microbiological and cytotoxicity assays and 65 x 10 x 2.5 mm for the three-point mechanical bending test.

We used muffle (Muffle no. 6, Jon Comércio de Produtos Odontológicos Ltda., São Paulo, SP, Brazil), which surfaces were isolated and filled with type III stone plaster (erodent; Vigodent SA Ind. Com., Rio de Janeiro, Brazil) on which the matrices were positioned, to wait the crystallization of the plaster. In order to facilitate the removal of the matrix during the disinclusion phase, silicone (Labor Mass, Vipi Ind. e Com. Ltda., Pirassununga, São Paulo, Brazil) was manipulated and positioned around it.

After the crystallization of the plaster, the muffle was opened and the matrix was removed and the space filled with the acrylic resin (Vipicril, Vipi Ind. e Com. Ltda., Pirassununga, São Paulo, Brazil), provided according to the manufacturer's recommendation (14 g of powder and 6.5 mL of liquid), added in proportion with 44 ppm silver nanoparticle aqueous solution (Khemia, SP, Brazil) at concentrations of zero, 1% (0.44 ppm), 2.5% (1.1 ppm) and 5%. (2.2 ppm) After this step the muffle was closed and the polymerization process was performed according to the protocol established by the manufacturer, with sequential thermal cycles of 70 ºC 30 min, 100 ºC 90 min, and subsequent cooling to 40 ºC).

2.3 Preparation of solutions

Concentrations were achieved as percentage from a 44-ppm AgNP stock solution. The solution was obtained by a physicochemical method of electrolysis. (Khemia, SP). In the process of obtaining AgNPs Khemia®, two pure silver electrodes, commonly called "thousand silver", with 99.99% purity, are immersed in distilled water and an alternating voltage of 1.5 V is used.

2.4 Assessed groups

Considering the application condition of silver nanoparticles with 44 ppm and concentration, 162 specimens were evaluated, 27 in each group as follows:

- **G1:** Thermopolymerized acrylic resin with an addition of 1% in the mass with aqueous silver nanoparticle solution;
- **G2:** Thermopolymerized acrylic resin with an addition of 2.5% in the mass with aqueous silver nanoparticle solution;
- **G3:** Thermopolymerized acrylic resin with an addition of 5% in the mass with aqueous silver nanoparticle solution;
- **G4:** Thermopolymerized acrylic resin immersed for 10 min in aqueous silver nanoparticle solution;
- **G5:** Thermopolymerized acrylic resin immersed for 24 hours in aqueous silver nanoparticle solution;
- **GC:** As control, we used thermopolymerized acrylic resin samples without any silver nanoparticle treatment.

After the immersion time, excess AgNP from the specimens of groups G4 and G5 was removed.

All specimens used for cytotoxicity and antifungal assays were previously sterilized with ethylene oxide (Esterilize Complexo de Serviços de Esterilização LTDA, Bahia, Brazil).

2.5 Microbiological assay

Candida albicans (ATCC18804) were aerobically grown on Sabouraud Dextrose Agar (SDA; Difco, Detroit, Michigan, USA) for 24 h at 36 ± 1° C. Yeast cells were inoculated into a Nitrogen Yeast Base (YNB; Difco, Detroit, Michigan, USA), supplemented with 100 mM glucose and aerobically incubated with shaking at 36 ± 1° C. The inoculum was prepared in YNB medium and optically standardized to a measurement of 107 cells / ml (OD = 0.25 at 520 nm). Sterile samples (n = 18) were immersed into their own environment and incubated in a 5% CO2 atmosphere for 24 h (TE399 CO2 incubator, Tecnal, Piracicaba, São Paulo, Brazil) at 36 ± 1ºC to promote microorganism growth. Afterward, the discs were carefully washed in phosphate buffer solution (PBS) and placed in a 24-well culture plate. Metabolic activity was determined with an XTT assay protocol. An XTT solution (PBS supplemented with 200 mM glucose, 1 mg / ml XTT and 0.4 mM menadione) was added to the wells, protected from light and incubated for 3 h at 37° C. Discs were removed and incubated in Falcon tubes containing 1 mL dimethylsulfoxide - DMSO (Sigma, St. Louis, Missouri, USA). After centrifugation, the supernatant was analyzed with a 492 nm spectrophotometer (Epoch).

2.6 Cytotoxicity assay

For this assay, a mouse fibroblast cells (3T3-E1) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) was used. Cell cultures were maintained in DMEM / F-12 basal medium (LGC Biotechnology, Sao Paulo, SP), supplemented with 10% fetal bovine serum (BFS, LGC Biotechnology, Sao Paulo, SP, Brazil) and 1% of antibiotic-antimycotic solution (10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml in 0.9% sodium chloride; Sigma, St. Louis, MO, USA) in a 95% humidity and 5% CO^2 at 37ºC.

Sterile samples (n = 54) were immersed in the medium and cell viability was assessed by 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, Missouri, USA) after 24, 48 and 72 h. After incubation in the samples, cells were incubated with medium containing MTT (5 mg / ml) for 3 h. The MTT solution was then aspirated and 200 µl DMSO (Sigma, St. Louis, Missouri, USA) was added. The plates were then shaken on a shaker plate for 5 min and 150 mL of this solution was transferred to a new 96-well plate. Optical density was read at 590 nm in the plate reader (Epoch, BioTek, Winooski, VT, USA), and data were expressed as absorbance. All experiments were repeated three times under the same conditions.

2.7 Mechanical testing

The three-point bending test was performed on a universal mechanical testing machine (Oswaldo Filizola AME 2kN, São Paulo, Brazil) with a 50 Newtons (N) load cell, and the applied loading speed was 5.0 mm. / min The specimens (n = 90) were placed on a support with 21 mm between the bases. A chisel-shaped device was fitted to the upper part

of the machine which served to compress the sample. The machine exerted compression to the specimen until its fracture. Values were expressed in Newton (N). Statistical Analysis

Initially, descriptive analysis (mean and standard deviation) was performed and the Shapiro Wilk test was used to test normality distribution of the data. To compare the means between the groups with normal distribution, the One-Way ANOVA test was used, followed by Tukey's posttest. For variables without normal distribution, the Kruskal-Wallis tests were used, followed by the Mann-Whitney test. The significance level adopted was 5% and the data were analyzed in the Statistical Package for Social Sciences for Windows (SPSS, version 21.0).

2.8 Statistical analysis

Initially, descriptive analysis (mean and standard deviation) was performed and the Shapiro Wilk test was used to test normality distribution of the data. To compare the means between microbiological groups, the One-Way ANOVA test was used, followed by Tukey's post-test (normal distribution). The comparison between cytotoxicity groups and time, the Kruskal-Wallis tests were used, followed by the Mann-Whitney test (non normal distribution). The significance level adopted was 5% and the data were analyzed in the Statistical Package for Social Sciences for Windows (SPSS, version 21.0).

3. Results and Discussion

Prosthetic stomatitis is a multifactorial infectious disease involving factors related to the microorganism and the host[6]. Such factors contribute to the manifestation of the disease that affects a significant portion of dental users[15]. In this sense, developing new therapies that reduce their appearance is the yearning of the dental community.

Thermopolymerizable acrylic resin is the most widely used material for manufacturing definitive prostheses as it has a combination of favorable characteristics such as ease of laboratory handling, light weight, inexpensive manufacturing, stability in the oral environment, aesthetics, proper staining and lack of toxicity[16]. However, thermopolymerizable acrylic resin itself has porous surface properties that allow Candida albicans to adhere and colonize the surface and develop into a biomass[16].

3.1 Microbiological assay

Table 1 shows the results of the microbiological assay. It is observed that the addition of nanoparticles during polymerization at all concentrations tested (G1, G2, G3) reduced the number of microorganisms, however without statistically differing from the control group (GC) ($p<0.05$). The groups immersed in nanoparticles (G4 and G5) showed larger microbiological reductions without being statistically different from each other ($p <0.05$). However, they differed from the control group (CG), and from G1 and G2 groups ($p> 0.05$).

A possible method for preventing or reducing Candida albicans adhesion to the inside of the prosthesis would be to modify the prosthesis-based resins by adding antimicrobial agents. Silver has long been recognized for its broad antimicrobial property[17]. Thus, the present study aimed to evaluate the antifungal efficacy of microwave polymerized acrylic resins added or immersed in silver nanoparticles, as well as to evaluate the cytotoxic effect on direct contact cells in the mucosa, allied to the mechanical analysis of these materials. AgNPs have been used for their antimicrobial effect in different biomedical applications[18] and can eliminate all pathogenic microorganisms[19].

Microbiological analysis showed that the addition of nanoparticles during polymerization, at all concentrations tested, showed lower microbiological reduction when compared to the immersion conditions. This could lead to a question as to whether the thermopolymerization process of the acrylic resin would inhibit or diminish the effect of AgNP. However, AgNP obtainment and acrylic resin thermopolymerizing are performed at the same temperature, approximately 100 ºC[20]. According to Wady et al[1], silver nanoparticles are retained in the acrylic resin network and their release to the aqueous environment is restricted, which we can associate with the low antifungal action of groups G1, G2 and G3. The groups that were immersed in AgNP solution obtained the best microbiological results, however the G5 group would need to be immersed for 24 hours while G4 would only need 10 minutes, a condition in which G4 presents the most relevant values for clinical use. In groups G4 and G5, in which the acrylic plates were immersed in the solution, the AgNP on their surface immediately contacted the fungi, which would explain the results obtained.

Another factor that could influence antimicrobial activity would be nanoparticle size. In the current study, a concentration of 44 ppm AgNP with an average size of 50 nm was used. It has been reported that the smaller the particles, the greater the antimicrobial effect[22,23], due to the larger surface area that interact with microorganisms[22]. Although nanoparticles smaller than 10 nm are better internalized by microorganisms[15] may have a greater cytotoxic and genotoxic effect on mucosal cells[14,24]. In this sense, Kirmanidou et al. (2019)[25] evaluated the microbiological effect on periopathogens and cytotoxicity with AgNPs sizes of 5 and 30 nm, showing that the 5 nm size had lower antifungal potential on periopathogens.

3.2 Cytotoxicity assay

Table 2 shows the results of the cytotoxicity assay. It is observed that at times 24h and 48h, groups G1, G2 and G3 had

Table 1. Viability assay of *Candida albicans*, treated under different conditions.

Groups	Average Mean and Standard Deviation (absorbance)
CG	0.09 (0.004) ^{A}
G1	0.07 (0.007) ^{AB}
G2	0.08 (0.007) ^{ABC}
G3	0.08 (0.005) ^{ABD}
G4	0.06 (0.004) ^{DE}
G5	0.06 (0.005) ^{EF}
p-value	≤ 0.001*

G1: Thermopolymerized acrylic resin plus 1% AgNP, **G2**: Thermopolymerized acrylic resin plus 2.5% AgNP, **G3:** Thermopolymerized acrylic resin plus 5% AgNP, **G4**: Thermopolymerized acrylic resin immersed for 10 min in AgNP, **G5**: Thermopolymerized acrylic resin immersed for 24 h in AgNP. * One-Way ANOVA test: different letters represent significant difference between groups ($p \leq 0.05$), Tukey post hoc test.

Table 2. Cell viability (absorbance) assay of fibroblasts treated under different conditions.

Groups	Mean and Standard Deviation			P-value#
	24h	48h	72h	
GC	0.52 (0.02)$^{a\ A}$	0.76 (0.06)$^{b\ A}$	0.55 (0.01)$^{a\ A}$	0.037
G1	0.28 (0.03)$^{ab\ B}$	0.35 (0.02)$^{a\ B}$	0.22 (0.05)$^{b\ B}$	0.019
G2	0.27 (0.02)$^{a\ BC}$	0.35 (0.01)$^{b\ BC}$	0.05 (0.00)$^{c\ C}$	0.023
G3	0.28 (0.03)$^{a\ BCD}$	0.36 (0.02)$^{b\ BCD}$	0.05 (0.00)$^{c\ CD}$	0.022
G4	0.13 (0.02)$^{a\ E}$	0.18 (0.07)$^{a\ E}$	0.26 (0.04)$^{b\ BE}$	0.039
G5	0.15 (0.08)$^{a\ EF}$	0.14 (0.08)$^{a\ EF}$	0.22 (0.03)$^{b\ BEF}$	0.117
p-value#	0.005	0.005	0.006	

G1: Thermopolymerized acrylic resin plus 1% AgNP, **G2**: Thermopolymerized acrylic resin plus 2.5% AgNP, **G3**: Thermopolymerized acrylic resin plus 5% AgNP, **G4**: Thermopolymerized acrylic resin immersed for 10 min in AgNP, **G5**: Thermopolymerized acrylic resin immersed for 24 h in AgNP. # Kruskal-Wallis Test: Different capital letters represent significant difference between groups ($p \leq 0.05$) for each time (vertical line). Different lower case letters represent significant difference ($p \leq 0.05$) for each group at different times (horizontal line), U Mann-Whitney Test.

lower cytotoxicity compared to groups G4 and G5 ($p < 0.05$). When 72 h time was evaluated, it was observed that groups G2 and G3 presented higher cytotoxicity than groups G1, G4 and G5 ($p<0.05$). However, at all times, all groups presented lower viability when compared to the control group ($p> 0.05$).

Thus, the use of AgNP associated with dental materials show high toxicity, but at low concentrations, up to 1%, its use is considered safe[26,27]. However, in this study, it was observed that under all conditions studied there was a higher cytotoxicity of the nanoparticle, regardless of the inclusion condition during polymerization or when compared to the control. Studies have shown that incorporation of AgNPs does not promote deleterious cellular effects[15,28], but it is important to consider beyond the above conditions, time and nanoparticle shape, condition and incorporation material[14,24,25]. Ahlberg et al.(2016)[29] highlighted a certain effect of AgNPs in reducing activity and viability of human skin cells and may be mutagenic or still influence inflammation mediators provoking of systemic responses, including toxicity, teratogenic or carcinogenic effects

Additionally, cytotoxic effects are more evident when nanoparticles are incorporated into thermopolymerized resin as compared to immersion, especially at the most advanced times (48 and 72h) and higher concentrations. In the immersion condition, the most damaging cellular effects are in the first 24 h, which emphasizes that the decrease of cytotoxic potential must occur by the decrease of AgNP film on the acrylic resin surface. Silver nanoparticle is very diffuse in aqueous medium[30]. It can be assumed that the decrease in cytotoxic potential of immersed samples (G4 and G5) over time occurs because of high diffusion of silver nanoparticle. Clinically, what can be thought is that after 10 minutes of immersion, proven antifungal effect, the plates could be washed in running water to eliminate AgNP leaving them with low cytotoxicity. Further studies should be performed to prove this hypothesis. In addition, it is important to highlight the more toxic effects of AgNP when incorporated at higher concentrations when observed in more advanced times, which corroborates again that release is initially difficult, unlike immersion, whose release and action is more labile.

3.3 Mechanical assay

For mechanical strength, the analysis of variance showed a statistically significant difference between the groups ($p<0.001$) (Table 3). The lowest resistance values were observed in the group with 5% incorporation of silver nanoparticle (G3). The G4 group immersed for 10 min did not differ statistically from the control group (CG), G1 and G5 ($p < 0.05$).

Table 3. Flexural Strength Test. (Strength in Newton).

Groups	Maximum Strength for Fracture Mean Average and Standard Deviation
CG	73.50 (6.88) ^{A}
G1	71.61 (7.78) $^{B\ A}$
G2	61.16 (9.31) ^{C}
G3	49.87 (6.41) ^{D}
G4	70.56 (5.98) ^{EAB}
G5	65.25 (9.06) ^{FBCE}
p-value	$\leq 0.001^{\#}$

G1: Thermopolymerized acrylic resin plus 1% AgNP, **G2**: Thermopolymerized acrylic resin plus 2.5% AgNP, **G3**: Thermopolymerized acrylic resin plus 5% AgNP, **G4**: Thermopolymerized acrylic resin immersed for 10 min in AgNP, **G5**: Thermopolymerized acrylic resin immersed for 24 h in AgNP. # Kruskal-Wallis Test: different letters represent significant difference between groups ($p \leq 0.05$), U Mann-Whitney Test.

Mechanical results revealed that 5% AgNP incorporations showed a reduction in flexural strength. The incorporation of AgNP into acrylic resins acts as impurity, increasing the potential for residual monomers not to leach from the surface[31], and consequently, the stress concentration area, resulting in mechanical properties loss[32]. According to Sehajpal & Sood (1989)[33], from 5% concentration there is a decrease in the mechanical properties of acrylic resins. Having in mind that according to the International Organization for Standardization (ISO 1957)[34], the minimum flexural strength for acrylic resins should be 65N. The results of the current study make it impossible to incorporate 5% AgNP, clinically representing a decrease in longevity due to early fractures.

It was observed that incorporation or immersion in AgNP of thermopolymerizable resin did not show satisfactory results for cytotoxicity, especially at higher concentrations. However, when the flexural strength test is observed, only the 5% incorporated acrylic resin changed. In the microbiological assay, samples immersed in silver nanoparticle significantly decreased the amount of microorganisms.

4. Conclusions

With this study, it is concluded that:

- thermopolymerizable acrylic resin immersed in silver nanoparticles decreased the amount of Candida albicans;
- the silver nanoparticle used is cytotoxic to fibroblasts;
- the incorporation of 5% silver nanoparticle decreased the flexural strength of acrylic resins, although when immersed no changes occurred.

5. References

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