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Antibacterial effects and cytotoxicity of an adhesive containing low concentration of silver nanoparticles

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ABSTRACT

Objectives: To evaluate the antibacterial effects, cytotoxicity and microtensile bond strength of an adhesive containing low concentrations of silver nanoparticles (NAg).

Methods: Various concentrations of NAg (50, 100, 150, 200 and 250 ppm) were incorporated into the primer of the Scotchbond Multi-Purpose adhesive system (SBMP). Antibacterial activity was examined using a broth microdilution assay to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), agar diffusion assay and the MTT assay was used to examine the biofilm metabolic activity (*S. mutans*). The Microtensile Bond Test (μTBS) was performed after 24 h, followed by 6-months storage in distilled water. Cytotoxicity was assessed with an MTT reduction assay in human dental pulp stem cells viability after exposure to Nag-conditioned culture media during 0, 24, 48, and 72 h. The results were statistically analyzed ($\alpha \leq 0.05$).

Results: MIC was found between NAg 25 and 50 ppm MBC was determined at 50 ppm of NAg. Bacterial activity inhibition was higher than control in all NAg groups compared to control in agar diffusion assay. Biofilm inhibition was statistically higher in 250 ppm NAg than control. All NAg groups and SBMP presented similar cytotoxicity in each period. Adhesives with NAg 200 and 250 ppm and SBMP (control) presented the highest μTBS values, similar to that of SBMP control, in both instances (24 h and 6 months) ($p > 0.05$).

Conclusions: The commercial primer containing NAg 250 ppm showed both antibacterial effect and reliable bond strength with no cytotoxicity increase. The addition of NAg to primers seems promising for the improvement of conventional dental adhesives efficacy.

Clinical Significance: The addition of low concentrations of NAg (250 ppm) to primers were effective to improve antibacterial effect preserving the bond strength and the biocompatibility of the commercial product. NAg/primer association could protect the tooth-adhesive interface increasing dental restoration longevity.

1. Introduction

The adhesion of dental materials to hard dental tissues continues to be a challenge for strong and durable bonding achievement in dental composite restorative procedures. Due to its rather complex nature, the tooth/restoration material interface is the most vulnerable point of an adhesive technique regardless of employed strategy, as is it subject to several conditions that may favor deterioration [1]. Stress may be caused by the composite material post-gel shrinkage during polymerization and by mechanical fatigue during mastication, generating micro-gaps that may weaken the restoration and eventually cause its failure [2]. In addition, interface degradation and adhesive failure may be accelerated by chemical events such as the polymeric matrix hydrolysis and the activation of metalloproteinases after collagen

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exposure [3,4], as well as the action of remaining bacteria from the infected dentin or from marginal infiltration [4].

The addition of components to commercial adhesives to protect collagen fibers exposed during etching have been studied with reasonable results [3]. Nevertheless, marginal infiltration of bacteria still remains an issue and several approaches to solve this matter have been proposed. The addition of antimicrobial agents to resins and adhesive materials, such as the use of antibacterial monomers [5,6], chlorhexidine [2,3,7], silver nanoparticles [8–10], and associations [5,6,11] have been extensively tested.

Silver nanoparticles (NAg) may be added to different dental materials [10], such as primers and adhesives [6,11], glass ionomers [12], acrylic resins [8], and orthodontic resins [13]. In fact, the NAg exhibits a wide antimicrobial spectrum, especially for bacteria [14–17]. One of the most commonly accepted mechanism of action for NAg is based on the cell membrane rupture upon contact with a silver metal surface, and the formation of reactive oxygen species upon NAg uptake or ion penetration into the nucleus, and the interaction with DNA inhibiting cell reproduction [15,18].

Preparation of dental materials containing homogeneously dispersed NAg is of relevance. Agglomeration and/or precipitation, as well as the available nanoparticle surface are important to define the antimicrobial and physical-chemical properties of the tooth/restoration material interface [19]. When NAg was dispersed in the polymeric matrix, promising results were achieved without compromising the materials' main physical-chemical properties [20]. Most studies on NAg incorporation in adhesive materials used high concentrations [5,6,11,21,22], usually 500 ppm (0.05%) and 1000 ppm (0.1%), neglecting the possibility of positive lower range effects.

The present study evaluated the effect of low NAg incorporation concentrations (in 50 ppm increments from 50 to 250 ppm) into the primer of a 3-step, commercially available etch-and-rinse adhesive system. Nanoparticles functionalized with suitable organic stabilizers that control particle sizes and reduce agglomeration were successfully prepared and added to the primer producing a hydrophilic and functional material even at low NAg concentrations. The purpose was to determine an optimal NAg concentration, which would allow for antibacterial effect without increasing the material's cytotoxicity and without compromising bond strength.

2. Materials and methods

2.1. Synthesis of NAg and incorporation into the primer material

Silver nanoparticles (NAg) were prepared using chemical methods, by reducing silver nitrate and using methods adapted from classical procedures including the citrate [23], borohydride [24], and the polyol method [25]. Typically, a 1×10^{-3} mol dm^{-3} $AgNO_3$ solution was refluxed for 5 min, then a 1% m/m sodium citrate and polyvinyl alcohol solution was added and the reaction mixture refluxed for additional 15 min. The relative amount of citrate and polymers (polyvinyl alcohol in ethanol) were adjusted to control nanoparticles size (5 to 50 nm) and the colloidal stability. Therefore, fully dispersible 20-nm average diameter NAg was successfully prepared and used as the primer additive. The dispersions were centrifuged at 14,000 rpm for 5 min and filtered through 0.45 and 0.22 μm polyvinylidene difluoride filter to remove eventual agglomerates. Silver nanoparticles were characterized by transmission electron microscopy (TEM). The experimental groups were defined by incorporating NAg colloidal dispersion directly to SBMP (Scotchbond™ Multi-Purpose, 3 M ESPE, St. Paul, MN, USA) primer, generating the control and experimental samples: SBMP (control – no NAg), NAg 50 ppm, NAg 100 ppm, NAg 150 ppm, NAg 200 ppm, and NAg 250 ppm.

2.2. Antibacterial effects: broth microdilution assay and agar template diffusion assay

2.2.1. Bacteria

All procedures were performed under sterile conditions. *S. mutans* (ATCC 25,175™, Microbiologics™, St. Cloud, MN, US) was used in the assays. Bacteria reactivation was carried out according to manufacturer's instructions. Bacteria were cultivated in Petri dishes containing growth medium (Brain Heart Infusion, BHI, Oxoid) and incubated for 48 h at 37 °C. Bacteria were suspended in saline solution at 0.5 McFarland concentration.

2.2.2. Brain heart infusion (BHI) culture medium preparation

Brain-heart infusion broth and agar were prepared with Milli-Q grade water (Millipore, Diadema, SP, Brazil), according to manufacturer's instructions. Fifteen mL of sterile (121 °C for 15 min) medium was added to Petri dishes.

2.2.3. Count of colony forming units (CFU)

A serial decimal dilution was performed with the 0.5 McFarland suspension. One mL of suspensions from dilutions were transferred to 15 mL of BHI agar medium in Petri dishes, in duplicate. Dishes were incubated at 37 °C, for 48 h. After that, bacteria colonies were calculated with the assistance of a colony counter (CP600 Plus, Phoenix Luterco, Phoenix Ind., Brazil).

2.2.4. Microdilution Broth Assay and determination of the minimal inhibitory and bactericidal concentrations

This technique was used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) using six different concentrations of NAg + primer (50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.13 ppm e 1.56 ppm) against *S. mutans* bacterial suspensions. 190 μL of a bacterial suspension was added to each well of the 96-well microplates. Next, 10 μL of samples were added to the corresponding wells. Each plate was set up with a positive (saline solution) and a negative (primer without NAg) bacterial growth control. The microplates were incubated for 48 h at 37 °C. MIC value was determined by visual examination; the absence of turbidity or a button of growth at the bottom of the well was defined as growth inhibition. Bacteria of all culture plate wells were submitted to the subculture in BHI agar medium incubated at 37 °C for 48 h. The MBC value corresponded to the well with the minimum NAg concentration that prevented bacterial growth was determined.

2.2.5. Agar template diffusion assay

This experiment followed the methodology suggested by CCLS (*Committee for Clinical Laboratory Standards*). Bacterial suspension (*S. mutans*) with 0.5 McFarland was used to inoculate the Petri dishes. Sterile swabs were used to seed bacteria onto the Petri dishes. The experimental groups containing NAg + primer at the various mentioned concentrations were tested as well as the control group (SMBP without NAg). The template was positioned over each dish and 10 μL were added to each group (n = 3). The dishes were incubated at 37 °C for 48 h. The growth diameter inhibition zones were horizontally and vertically measured with a digital caliper (Mitutoyo Corp., Kawasaki, Japan).

2.2.6. Biofilm inhibition assay

The specimens (5 mm × 1 mm) were made using: primer + adhesive + composite resin (Z350 XT, 3M ESPE, St Paul, USA). The primer was firstly applied to the matrix, dried for 5 s and then the adhesive was applied and photoactivated for 10 s. The composite resin was inserted into the matrix in a single increment and photoactivated for 20 s. The specimens were sterilized with ethylene oxide.

Culture method was followed according to the preconized by Castilho et al. [26] with adaptations related to the bacteria growth