Chitosan incorporated in a total-etch adhesive system: antimicrobial activity against *Streptococcus mutans* and *Lactobacillus casei*

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The aim of this study was to investigate the antimicrobial effect of an experimental adhesive system containing chitosan (0.2% and 0.5%) against *Streptococcus mutans* and *Lactobacillus casei.* Twenty-four extracted human third molars were used, and 4 cavities were prepared in each tooth. The teeth were contaminated with either *S mutans* (n = 12 teeth, 48 cavities) or *L casei* (n = 12 teeth, 48 cavities) microorganisms. One cavity in each tooth received 1 of the following treatments: negative control (no treatment [NC]), positive control (Adper Single Bond 2 [SB]), an experimental adhesive containing chitosan 0.2% (CHI2), or an experimental adhesive containing chitosan 0.5% (CHI5). After sealing of the cavities and an incubation period, dentin scrapings were collected from each cavity for microbiological evaluation. Analysis of variance and Tukey tests revealed no statistically significant differences among the SB, CHI2, and CHI5 groups ($P > 0.05$), but all 3 differed significantly from the NC group ($P \leq 0.05$). The concentrations of chitosan did not influence the antimicrobial effect against *S mutans* and *L casei,* presenting a similar effect to that of a conventional 2-step adhesive system.

Received: May 10, 2016
Accepted: June 20, 2016

Key words: chitosan, dentin, *Lactobacillus casei, Streptococcus mutans*

GENERAL DENTISTRY
SELF-INSTRUCTION
CE CREDIT
Exercise No. 399, p. 67
Subject code: Dental Materials (017)

The longevity of a composite resin restoration is directly related to the stability of the hybrid layer.[1-4] However, the methacrylate polymers of adhesive systems—such as bisphenol A glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), or hydroxyethyl methacrylate (HEMA)—may undergo chemical hydrolysis with an increase in the acidity of the monomer components. This leaves zones rich in water within the hybrid layer, causing either the elution of the residual monomer or enzymatic degradation by the metalloproteinases and salivary esterases, leaving the hybrid layer susceptible to hydrolysis.[1,5-7]

Inhibiting the action of these enzymes and preventing degradation of the hybrid layer requires the use of cavity pretreatments, adhesive systems resistant to the action of esterases, or collagenolytic enzyme inhibitors.[5,6,8] It is also necessary to preserve the structural integrity and mechanical properties of collagen fibers and increase their resistance to biodegradation.[6] In this context, the natural biopolymer chitosan has the capacity to form a microfibrillar and nanofibrillar network with superior mechanical properties. When this network is associated with bonding agents, it has the potential to show improved resistance to degradation of the mechanical properties of dentin.[9-12]

Chitosan is derived from chitin by deacetylation. Chitosan has versatile physicochemical and biological characteristics that allow its application in diverse areas, such as in genetic therapy, where it is used to repair breaks in the double strand of DNA.[9,13] Chitosan has been used to promote the biomimetic reconstruction of enamel and inhibit biofilm formation on titanium implant surfaces.[14,15] The incorporation of chitosan in experimental adhesive systems associated with methacrylate monomers has been suggested as a way to improve the biological and mechanical properties of collagen construction and enhance antibacterial activity by means of ionic interactions between chitosan and the bacterial cells.[16]

The antibacterial activity of chitosan occurs by means of an intrinsic mechanism, which results from a sequence of molecular events (such as ion exchange within the cell), the pH of the chitosan solution, and the constitution of the bacterial cell wall.[17,18] Positively charged glucosamine groups (NH_3) give chitosan a cationic nature that may be the fundamental contributory factor for its interaction with the negative charge of the bacterial cell surface. The antibacterial activity of chitosan is due to the electrostatic interaction between the cationic chitosan and the negatively charged bacterial cell surface. This interaction causes the bacterial cell wall to rupture.[16,18-20] This mechanism removes

Table 1. Adhesive systems used in the study.

Material	Composition	Manufacturer
Conventional 2-step adhesive system (Adper Single Bond 2 [SB])	Water, ethanol, silica nanoparticles, Bis-GMA, HEMA, dimethacrylates, functional copolymer of polyacrylic and polyalkenoic acid methacrylates	3M ESPE
Experimental adhesive system containing chitosan 0.2% (CHI2)	55% Bis-GMA, 45% HEMA, 0.7% camphorquinone, 0.7% DMAEMA, chitosan 0.2%	Sigma-Aldrich
Experimental adhesive system containing chitosan 0.5% (CHI5)	55% Bis-GMA, 45% HEMA, 0.7% camphorquinone, 0.7% DMAEMA, chitosan 0.5%	Sigma-Aldrich

Abbreviations: Bis-GMA, bisphenol A glycidyl methacrylate; DMAEMA, *N,N*-dimethylaminoethyl methacrylate; HEMA, hydroxyethyl methacrylate.

the lipoteichoic acid from the cell membrane of gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus simulans,* causing harm to the diffusion mechanism within the cell membrane and leading to interruption in bacterial activity.[19]

The low molecular weight of chitosan has also been reported to prevent the adsorption of *Streptococcus mutans* by hydroxyapatite, which may be beneficial when this component is incorporated in different dental materials. The different concentrations of chitosan that may be incorporated into the adhesive system must be evaluated.[21]

When incorporated in the primer of adhesive systems, some monomers, such as methacryloyloxy dodecylpyridinium bromide (MDPB), a quaternary ammonia, have a bacteriostatic effect against *S mutans* that inhibits bacterial leakage without affecting the adhesive capacity in both in vitro and in vivo conditions.[22] Another compound, methacryloxyethyl cetyl dimethyl ammonium chloride (DMAE-CB), unites with the matrix of the adhesive and has the characteristics of a bacteriostatic agent by contact.[22] However, these compounds do not repair DNA structure after damage is caused by the release of methacrylate monomers from incompletely polymerized adhesive systems. This makes chitosan—which has been shown to repair DNA damage—superior for molecular repair when incorporated in adhesive systems.[13]

The aim of the present study was to analyze the antimicrobial effect of chitosan—incorporated in an experimental conventional 2-step adhesive system—against the bacteria *S mutans* and *Lactobacillus casei.* The experiment tested 2 null hypotheses: there would be no differences between the different concentrations of chitosan added to the conventional adhesive system with regard to their antimicrobial effects against *S mutans* and *L casei;* and there would be no differences between the antimicrobial effects of conventional adhesive systems, either containing chitosan or not, against *S mutans* and *L casei.*

Materials and methods

Tooth selection and cavity preparation

The project was approved by the Research Ethics Committee of the São Leopoldo Mandic Dental School and Research Center, Campinas, Brazil (protocol 32497014.3.0000.5374). A total of 24 healthy, extracted human third molars, stored in an aqueous 1% thymol solution for a period not exceeding 6 months, were used in this study.

After the teeth were cleaned with periodontal curettes and a Robinson brush (Microdont), the occlusal enamel was removed, exposing superficial dentin perpendicular to the long axis of the tooth. Four cylindrical cavities were prepared in the dentin surface without exposing pulp. A cylindrical diamond tip (No. 2292, KG Sorensen) was used to obtain cavities measuring 2 mm in diameter and 2 mm deep.[23,24] The teeth were then randomly divided into 2 groups (n = 12) according to the microorganism to be evaluated.

Microbial contamination and cavity treatments

Strains of microorganisms of the *S mutans* (ATCC 25175) and *L casei* (ATCC 393) types were acquired. From the lyophilized cultures, stationary phase cultures were prepared. The strains were kept under refrigeration and activated 24 hours before being used. This activation was performed in specific culture broths (brain-heart infusion broth for *S mutans* and de Man, Rogosa, and Sharpe [MRS] broth for *L casei*).

After the cultures were activated, the teeth, previously autoclaved for decontamination of the cavities, were placed in tubes with strains of each microorganism and then kept in an oven at a temperature of 36°C for 72 hours. After incubation was completed, the cavities of each tooth were assigned to one of the treatments: negative control (no treatment [NC]), positive control (Adper Single Bond 2 [SB]), an experimental adhesive containing chitosan 0.2% (CHI2), or an experimental adhesive containing chitosan 0.5% (CHI5). The composition of each material is detailed in Table 1.

Before the treatment was applied to the other cavities, dentin scrapings collected from the pulp walls and the surroundings of the NC cavity of each tooth were collected to certify that all the cavities in dentin were contaminated. To standardize the quantity of dentin scrapings collected for microbiological analysis, a pilot study had been previously conducted, so that the same procedure could be applied in all collections. A No. 2 carbide bur (KaVo Bur - carbide, KaVo Dental) was coupled to a contra-angle reducer (contra-angle reducer KaVo 20:1, KaVo Dental) and low-speed micromotor (Micromotor KaVo Intra, KaVo Dental). The bur was put in the cavity, and the contra-angle reducer was activated; the bur was moved around inside the cavity in 5 turns for complete removal of the dentin scrapings. A dentin excavator (Golgran Instrumentos Cirugicos e Odontológicos) was used to remove the scrapings and place them in a container holding 500 µL of sterile saline solution.

Table 2. Mean (SD) counts of *Streptococcus mutans* and *Lactobacillus casei* in treated cavities.

Treatment	Bacteria (CFU/mL)	
	Lactobacillus casei	*Streptococcus mutans*
NC	3.2×10^6 (2.2×10^6)[a]	6.8×10^5 (6.6×10^5)[a]
SB	0.9×10^6 (0.8×10^6)[b]	0.3×10^5 (0.7×10^5)[b]
CHI2	0.9×10^6 (0.8×10^6)[b]	1.3×10^5 (3.1×10^5)[b]
CHI5	0.7×10^6 (0.7×10^6)[b]	1.5×10^5 (3.3×10^5)[b]

Abbreviations: CFU, colony-forming unit; CHI2, experimental adhesive containing chitosan 0.2%; CHI5, experimental adhesive containing chitosan 0.5% ; NC, negative control (cavity without treatment); SB, positive control (conventional 2-step adhesive system [Adper Single Bond 2]).

Means followed by different lowercase superscript letters within columns are significantly different ($P \leq 0.05$).

The NC cavity of each tooth was identified with a marker. In the SB cavities, a light jet of air was applied for 5 seconds with the aid of a pipette, and 2 coats of a conventional 2-step adhesive system (Adper Single Bond 2) were applied with a disposable microbrush with a thin tip (Cavibrush, FGM Produtos Odontológicos) so that the adhesive would remain inside the cavity without flowing out. The adhesive was gently agitated for 15 seconds with a disposable microbrush while a light jet of air was applied with the aid of a pipette, thus preventing the adhesive from flowing out of the cavity. Light activation for 10 seconds was performed with a halogen unit (Demetron LC Optilux 500, Kerr Corporation) with a mean intensity of 450 mW/cm^2. The light intensity was measured with a radiometer (Newdent) after every 3 light activations.

The CHI2 cavities received an application of the experimental adhesive system with chitosan 0.2%. The CHI5 cavities received an application of the experimental adhesive system containing chitosan 0.5%. Each system was applied with protocol similar to that used for the SB cavities.

After the treatments, each cavity was sealed with a sterilized absorbent paper disc (2-mm-diameter Melitta paper filter, Mellita USA) placed in the most superficial area of the cavity.[25] The cavities were sealed with composite resin (Filtek Z350, 3M ESPE). The resin was applied carefully only on the paper disc and around the cavity. The composite resin was light activated for 20 seconds. The teeth for each incubation medium were kept separately in an oven with a 5% carbon dioxide atmosphere at 36°C ± 1°C for 72 hours.

Opening of cavities and dentin scraping collection

After the storage period, the temporary composite resin seal was removed with a sterile dentin excavator used with light pressure. Dentin specimens were collected, as previously described, for all the cavities that received treatment, and microbial cultures were performed. For this purpose, the flasks containing the dentin specimens were agitated in an agitation tube (AP 56, Phoenix Luferco) for 1 minute to disperse the bacterial aggregates. The solution was then agitated for an additional 20 seconds to guarantee homogeneity and decimal dilution from 10^{-1} to 10^{-2}. After this, aliquots of 10 µL of each dilution were spread onto solid media: MRS agar for *L casei* and mitis salivarius agar complemented with sucrose (20%), 0.2 U/mL of bacitracin, and 1% potassium tellurite (MSB) for *S mutans*. The plates with MSB and MRS agars were incubated in a 5% carbon dioxide atmosphere at 36°C ± 1°C for 48 hours.

Subsequently the colony-forming units (CFU) per milliliter were counted according to the morphologic characteristics of *S mutans* and *L casei*.

Statistical analysis

Exploratory data analysis indicated the need to transform the data into square roots so that the data (CFU/mL) would meet with the presuppositions of a parametric test. After the transformations, analysis of variance for the experimental design in random blocks and Tukey tests were applied at a level of significance of 5%. All analyses were performed with SAS version 9.2 software (SAS Institute).

Results

Contamination of the cavities with *S mutans* and *L casei* was verified, as the NC group presented a significantly greater number of CFU/mL ($P \leq 0.05$) than did the other cavities (Table 2). The SB cavities (positive control) showed a statistically significant difference from the NC group in regard to the quantity of CFU/mL. There was no statistically significant difference among the SB, CHI2, and CHI5 groups ($P > 0.05$).

Discussion

Inadequate sealing at the tooth-restoration interface may lead to microleakage, allowing penetration of microorganisms related to the onset and progression of caries.[26,27] Therefore, different strategies may be necessary to inhibit the development of carious lesions and promote suppression of any residual infection.[16,27]

Composite resins with antimicrobial characteristics have been incorporated in adhesive systems with the purpose of promoting inhibition of bacterial activity and thereby eliminating the risk of demineralization and secondary caries.[28] Agents such as MDPB, DMAE-CB, and chlorhexidine have been shown to present elevated antibacterial activity against cariogenic microorganisms.[22,28]

In the present study, the chitosan added to the experimental adhesive system showed an antimicrobial effect against *S mutans* and *L casei* similar to that of the adhesive system Adper Single Bond 2. Chitosan generates antimicrobial activity by exhibiting a high chelating capacity for metal ions present in the molecule of the cell wall of microorganisms. In addition, it presents a synergic effect when associated with other compounds, such as glass ionomer cement, amelogenin, riboflavin, soybean milk, and hyaluronic acid.[14,15,18,29,30] However, the present study demonstrated that the biopolymer chitosan promoted no greater antimicrobial effect than that presented by the use of a conventional adhesive system that did not have chitosan in its composition.

Although it was expected that the antimicrobial effect of chitosan would be directly proportional to its concentration in the experimental adhesive, the different concentrations of 0.2% and 0.5% exhibited no statistically significant difference with regard to antimicrobial potential. Thus, the null hypothesis concerning the influence of the concentration of chitosan against *S mutans* and *L casei* was not rejected. However, a study by Elsaka verified that a conventional 2-step adhesive system containing 0.12%-1.0% chitosan reduced the growth of *S mutans*.[16] This effect could be related to the pH of the adhesive because, as the concentration of chitosan increased, the pH of the adhesive diminished.

When comparing the antimicrobial effect of using a silver diamine fluoride composite containing 0.12% chlorhexidine with the effect of another material containing hydrated sodium borate and silver nitrate associated with 2.3% chitosan, Targino et al observed a statistically significant difference between the composites; better results against *S mutans* were obtained with the material with chitosan.[31] However, although greater antimicrobial potential has been accredited to chitosan in previous research, this effect was not observed in the present study, even when a higher concentration was used.

The adhesive Adper Single Bond 2 is a system with weak acid potential (approximately pH 4.5).[11,16] Some authors have suggested that this agent may have an antimicrobial effect before the polymerization of the material is initiated and the initial acidity could reduce bacterial growth.[11,16] In the present study, its antimicrobial effect against the studied bacteria was verified, since a lower number of bacterial colonies formed in SB-treated cavities. This suggests that the specific properties of the adhesive system, such as viscosity, pH, and the presence of monomers with different molecular weights, might have contributed to these results.[32] However, the concentrations of components such as HEMA have not been revealed by the manufacturer of Adper Single Bond 2 and may differ from those present in the experimental adhesive. Resin materials that contain HEMA exhibit substantial antibacterial activity.[33,34] The monomer HEMA has hydrophilic characteristics and may absorb water within the adhesive layer. It has been speculated that this might reduce the degree of conversion of the adhesive and potentiate the action of the monomers present in the adhesive, determining different degrees of biofilm formation produced by *S mutans*.[35,36] Based on the results of the present study, the other null hypothesis was not rejected, because there was evidence of antibacterial activity in all 3 adhesive groups.

Other physical and mechanical properties must be evaluated when a component is incorporated in an adhesive system, which should motivate further studies. The authors suggest the addition of chitosan to an adhesive system containing HEMA and ethanol could present good stability; there would be electrostatic interactions between the chitosan and the organic component of demineralized dentin composed of collagen and glycosaminoglycans, increasing the stability of the hybrid layer.[12] Reduction in nanoleakage could be observed at the interface of systems containing chitosan because of its chemical and physical interaction with the dentin substrate, an event not observed in systems without chitosan in their formulation.[12]

The results of the present study verified that both concentrations of chitosan in the experimental adhesive system inhibited the growth of microorganisms, similar to the conventional adhesive system. The authors recommend that further studies be conducted to explore the antimicrobial potential of this biopolymer and its influence on the bond strength to dental substrates after long periods of aging. In additions, future studies should evaluate different chitosan concentrations that may present greater antimicrobial effects.

Conclusion

Chitosan, added to an experimental adhesive system in concentrations of 0.2% and 0.5%, presented antimicrobial effects against *S mutans* and *L casei* in a manner similar to those of a conventional adhesive system, Adper Single Bond 2.

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Disclaimer

The authors have no financial, economic, commercial, or professional interests related to topics presented in the manuscript.

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