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Dental Materials

Influence of addition of [2-(methacryloyloxy)ethyl] trimethylammonium chloride to an experimental adhesive

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Abstract: The aim of this study was to develop an experimental adhesive with addition of [2-(methacryloyloxy)ethyl]trimethylammonium chloride (METAC) and to evaluate its mechanical and biological properties and its *in vitro* antibacterial activity. An experimental adhesive resin was formulated with Bis-GMA, TEGDMA, and HEMA. The antibacterial monomer was added at concentrations of 1%, 2.5%, and 5% (METAC groups). A group without METAC addition was used as control. The experimental adhesives were evaluated as to their antibacterial potential against *Streptococcus mutans*, degree of conversion, and softening in ethanol for 2 hours. The data were analyzed by one-way ANOVA, Tukey's post-hoc test, and the paired Student's t-test (significance level of 0.05). METAC showed antibacterial activity against *S. mutans* at all concentrations ($p < 0.05$). There was no statistical difference across METAC groups ($p > 0.05$). The 1%, 2.5%, and 5% groups yielded the highest mean values for degree of conversion ($p < 0.05$). The 1% group did not differ from the control group ($p > 0.05$). There was no statistical difference in baseline microhardness values ($p > 0.05$) and microhardness values after immersion in ethanol were lower than at baseline for all groups ($p < 0.05$). There was no statistical difference in the reduction of Knoop hardness number (KHN) after immersion in ethanol for any of the groups ($p > 0.05$). The results of the present study indicate that METAC is a promising antibacterial agent when added to an adhesive system.

Keywords: Anti-infective Agents; Adhesives; Dentin-Bonding Agents.

Introduction

Streptococcus mutans is linked to biofilm formation and accumulation, associated with dental caries etiology.[1] Some studies have shown that secondary caries is one of the primary reasons for restoration failure.[2,3] Thus, antibacterial agents have been added to adhesive systems as an effective dental treatment strategy.[4,5,6,7] The use of antibacterial agents leads to inactivation of bacteria and may hamper dental caries progression.[8] Furthermore, the antibacterial property of adhesive systems could bring an additional benefit to the conservative management of carious

lesions[9,10,11] by improving the success and longevity of restorations. Notwithstanding, there are not many *in vivo* studies demonstrating the clinical efficacy of antibacterial adhesives.[12]

Antibacterial agents can copolymerize or not with the resin matrix, which will determine the release of antibacterial agents.[13] Chlorhexidine, fluoride, and silver particles release antibacterial agents, and since it is impossible to control the kinetics of release, antibacterial activity decreases over time. Monomers with quaternary ammonium salts (QAS) are immobilized in the resin matrix by copolymerization with methacrylates.[14] QAS are an organic quaternary ammonium compound with nitrogen bound to four radicals.[15] Thus, the molecular structure of [2-(methacryloyloxy) ethyl]trimethylammoniumchloride (METAC) (Figure 1), which contains a quaternary ammonium group (antimicrobial functionalities), allows copolymerization with other methacrylate monomers and produces antibacterial polymers.[16] Therefore, METAC-containing materials could be incorporated into adhesive systems to inhibit bacterial activity, leading to long-term efficacy.[17]

Considering the short alkyl chain length of METAC, antimicrobial activity probably derives from the charged nitrogen atom of quaternary ammonium, which concentrates the positive charge.[18] QAS, just like METAC, may cause lysis of bacterial membranes. The positively charged sites of quaternary ammonium when in contact with negatively charged bacterial sites may disturb the electrical balance of the cell membrane. The bacteria could then be damaged or killed by cytoplasmic leakage.[19,20] QAS-containing bonding agents act upon the bacteria that are closest to the resin surface layer. This is related to the contact killing mechanism, whose QAS is copolymerized with and immobilized in the resin.[21] Chlorhexidine, an antibacterial, has shown some ability to help inhibit adherence of microorganisms to a surface, thereby preventing biofilm growth and development.[22] However, it is difficult to control the release from the comonomeric mixture, which reduces interface stability.[12] Moreover, a previous study showed that growth inhibitory effects against *S. mutans* from the release of silver ions may cause polymer degradation.[23]

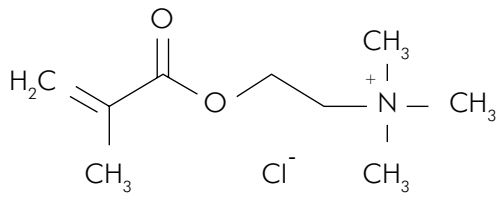

Figure 1. Chemical structure of [2-(methacryloyloxy)ethyl] trimethylammonium chloride (METAC).

The aim of this study was to develop an experimental adhesive by addition of METAC at different concentrations (1%, 2.5%, and 5%) and to evaluate *S. mutans* inhibition, degree of conversion, microhardness, and softening in ethanol. The null hypothesis is that the addition of METAC does not interfere with adhesive resin properties.

Methodology

Formulation of experimental adhesive resins

Experimental adhesive resins were formulated by mixing 50 wt.% bisphenol A glycidyl methacrylate (Bis-GMA), 25 wt.% triethylene glycol dimethacrylate (TEGDMA), and 25 wt.% 2-hydroxyethyl methacrylate (HEMA). METAC was added at four concentrations: 0, 1, 2.5, and 5 wt%. Camphorquinone, DMAEMA, and diphenyliodonium salt were added as initiator system to the matrix at 1 mol% according to the moles of monomers used. The initiators were mixed with monomers and ultrasonicated for 480 s. A light-emitting diode unit (Radii Cal, SDI LTD., Australia) was used for photoactivation with an irradiation value of 1,200 mW/cm^2 measured with a digital power meter (Ophir Optronics, USA). All reagents were purchased from Aldrich Chemical Co. (St. Louis, USA).

Direct contact inhibition

According to a previous study,[24] three cylindrical samples of adhesive (1 mm in height and 3 mm in diameter) were made for each group. The specimens were sterilized in hydrogen peroxide plasma. The gram-positive *S. mutans* (OMZ175) was grown aerobically in brain heart infusion (BHI) broth

(HiMedia Laboratories Pvt. Ltd, Mumbai, India) at 37°C. Cells were harvested by centrifugation and resuspended in a fresh medium. Inocula were prepared by adjusting the cell suspension to a predetermined optical density (OD) of 0.02 at 600 nm. Using a 96-well plate, each specimen was placed in a well with 300 µL of BHI broth. Each well was inoculated with 20 µL of the *S. mutans* suspension. The negative control consisted of three sets of wells containing uninoculated fresh medium (300 µL). Immediately and 24 hours after the placement of the inocula, 90 µL of each well content was diluted in saline to 10^{-8}. The 10^{-1}, 10^{-3}, 10^{-6}, and 10^{-8} dilutions were plated onto BHI agar using 25-µL aliquots of each dilution in duplicate. The plates were incubated at 37°C under anaerobic conditions. The colonies were counted visually after 24 hours and scaled by dilution factors and then transformed into colony-forming units (CFUs) per milliliter. The experiment was performed under aseptic conditions.

Degree of conversion

The experimental adhesive was evaluated using Fourier transform infrared spectroscopy (FTIR) with a Vetrex 70 (Bruker Optics, Ettlingen, Germany) spectrometer equipped with an attenuated total reflectance device composed of a horizontal diamond crystal with a mirror angle of 45 degrees. The spectrometer received a support for the light-curing unit and the 5-mm distance between the fiber tip and the sample was standardized. The Opus software (Bruker Optics, Ettlingen, Germany) used a Blackman-Harris 3-Term apodization function in a range of 4000 to 400 cm^{-1} and 64 scans with a 4cm^{-1} resolution. The samples were directly dispensed onto the diamond crystal into a polyvinyl siloxane matrix for standardization (5 mm in diameter and 1 mm in height). One spectrum was obtained before photoactivation and another one immediately after photoactivation for 20 s (n = 3). The degree of conversion (DC) was calculated according to a previous study,[25] considering the intensity of 1,635 cm^{-1} carbon-carbon double-bond stretching vibration (peak height) and using the 1,608 cm^{-1} aromatic carbon-carbon bond from polymerized and unpolymerized samples as internal standard.

Softening in ethanol

The same specimens produced during the evaluation of degree of conversion were used to determine solvent degradation. Three specimens for each experimental adhesive (n = 3) were embedded in acrylic resin and polished. Thereafter, they were stored and dried at 37°C for 24 hours. The specimens were subjected to a microhardness test in which five indentations (10 g/5 s), 100 µm apart from each other, were assessed using a digital microhardness tester (HMV 2, Shimadzu, Tokyo, Japan). Microhardness was calculated according to a previous study.[26] The initial Knoop microhardness number (KHN_1) was recorded, the specimens were subjected to softening in absolute ethanol at 37 °C for 2 hours, and the final post-conditioning hardness (KHN_2) was then determined. The percent reduction between KHN_1 and KHN_2 (ΔKHN%) was calculated.

Statistical analysis

The number of CFUs and degree of conversion were analyzed by one-way ANOVA and Tukey's post-hoc test. Softening in ethanol was assessed by the paired Student's t-test (KHN1 and KHN2) and ΔKHN% by one-way ANOVA. A level of significance of 0.05 was considered for all tests.

Results

The direct contact inhibition (DCI) values are shown in Figure 2. Addition of METAC reduced the number of CFUs compared with the negative control and experimental adhesive without METAC ($p < 0.05$). There was no statistical difference across METAC groups ($p>0.05$). The mean DCI values ranged from 72.6 to 74.5%. The 2.5% and 5% groups yielded the highest mean DCI values ($p < 0.05$). The 1% group did not differ from the control group ($p > 0.05$). Table shows microhardness values before (KHN_1) and after (KHN_2) immersion in ethanol, the percent reduction between KHN_1 and KHN_2 (ΔKHN%), and the degree of conversion. There was no statistical difference in initial microhardness values and difference in percent values between KHN1 and KHN2 for any of the groups ($p > 0.05$). Microhardness values after immersion in ethanol were lower than the initial ones for all groups ($p < 0.05$).

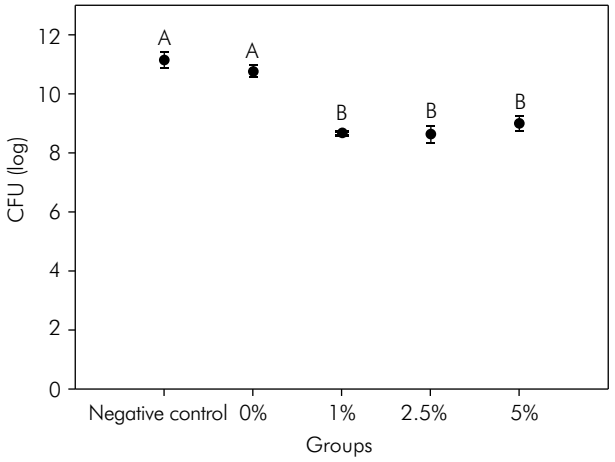

Figure 2. Figure 2. Median and 25th and 75th percentiles in CFU (log). Different uppercase letters indicate significant differences ($p < 0.05$).

Table. Microhardness values before (KHN1) and after immersion in solvent (KHN2) and the variation of microhardness values (Δ%). Mean (+- standard deviation) degree of conversion (DC).

Groups	KHN1	KHN2	ΔKHN%	DC (%)
0%	13.7 (±0.3)A,a	5.1 (±0.3)b	72.6 (±6.1)A	72.6 (±0.9)B
1%	13.3 (±0.4)A,a	4.8 (±1.1)b	74.5 (±3.1)A	73.2 (±0.6)B
2.5%	13.6 (±0.9)A,a	4.6 (±1.2)b	68.7 (±3.7)A	74.5 (±0.3)A
5%	11.9 (±2.7)A,a	2.8 (±0.5)b	76.8 (±4.8)A	74.2 (±0.4)A

Different uppercase letters in the same column indicate statistical difference ($p < 0.05$). Different lowercase letters in the same row indicate statistical difference between KHN1 and KHN2 ($p < 0.05$).

Discussion

Antibacterial adhesives have been introduced to allow the reduction of biofilm accumulation at the tooth/restoration interface and could provide long-term antibacterial activity.[12] In the present study, an experimental METAC-containing adhesive resin was used at three concentrations. All adhesives with this compound presented antibacterial activity against *S. mutans* and resistance to solvent degradation did not differ from that of the control group. Degree of conversion improved from 2.5 wt% with the addition of the antibacterial monomer. Thus, the null hypothesis had to be rejected.

A compound with a methacrylate functional group that copolymerizes with the comonomer mixture of the adhesive, preventing leakage and improving antibacterial activity over time, is required.[21,27] In this study, all groups showed antibacterial activity against *S. mutans* at different METAC concentrations compared to the negative control. METAC, when incorporated into an adhesive system, has the significant potential to inhibit biofilm formation, in addition to intrinsic anti-adhesive and detergent properties against gram-positive bacteria.[28] The antibacterial mechanism is probably due to contact of the negatively charged bacterial sites and positively charged QAS, diffusion through the cell wall, binding to the cytoplasmic membrane, and/or disruption of the cytoplasmic membrane, release of cytoplasmic constituents, and cell death.[29,30,31] METAC showed better results at lower concentrations than other antibacterial copolymers (*e.g.*, benzotriazole), which achieved antibacterial effect only at 5 wt%.[24] In this study, the *in vitro* antibacterial effect was not tested after a long period. Nevertheless, it was previously shown that copolymerization is responsible for growth inhibition of bacteria in contact with the adhesive for 6 months after polymerization using another antibacterial monomer [methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB)].[32] Thus, METAC is expected to have this behavior over time. However, it is imperative to evaluate antibacterial effect on other cariogenic microorganisms (*e.g., lactobacilli*).[12]

The number of unreacted monomers increases as the degree of conversion decreases, enhancing the chances of damage to the pulp and periapical tissues.[21] Incorporation of METAC was found to be advantageous, since the degree of polymer conversion increased for the experimental adhesive resin at the 2.5% and 5% concentrations. These findings indicate that incorporation of METAC had no adverse influence on the curing behavior of Bis-GMA/TEGDMA/HEMA-based adhesive resin. Although there is a statistically significant difference in the degree of conversion, its values corroborate those of the literature,[4] but it is not possible to state they will be clinically significant. METAC is a monomethacrylate[16] and there may have been fewer unreacted double bonds after the reaction, not indicating reduced polymerization, though. The degree of conversion is directly associated with the mechanical properties of polymers; however, it is not necessarily related to crosslink density.[33] Regarding long-term durability, it was reported that QAS containing reactive methacrylate

groups can be copolymerized and covalently bonded to the resin matrix upon photopolymerization.[4] This copolymerization immobilizes the antibacterial agent, preventing its leakage, which could result in material with poor physical and chemical properties.[12]

The softening test is an alternative method for crosslink density evaluation.[34] It is usually accepted that highly cross-linked polymers are more resistant to degradation and solvent uptake whereas linear polymers present more space and pathways for solvent molecules to diffuse within their structure.[35] The incorporation of METAC did not influence the initial microhardness values, as KHN1 was not different across the groups. The microhardness values observed in all experimental groups were not different from those of the control group after 2 hours of immersion in ethanol. METAC has one carbon-carbon double bond (Figure 1) whereas Bis-GMA and TEGDMA have two. This could result reduce crosslink density.[36] However, as softening in ethanol is assumed to reflect the crosslink density of a polymer,[37] METAC is a reliable alternative.

It is necessary to determine whether the addition of QAS to comonomer mixtures has some effect on their mechanical properties and does not compromise dentin bond strength.[4,5,6,7] The results of the present study indicate that METAC is a promising antibacterial agent when added to an adhesive system.

Conclusion

METAC copolymerized with adhesive resin exhibited antibacterial properties against *S. mutans*, proving to be beneficial to long-term restorations.

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