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Characterization of an antimicrobial dental resin adhesive containing zinc methacrylate

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Abstract This study evaluates the effect of zinc methacrylate (ZM) on the degree of conversion (DC), cytotoxicity and antimicrobial activity (AA) of an experimental resin. Tetraethyleneglycol dimethacrylate was used as the experimental resin and it was photo activated using camphoroquinone and ethyl 4-dimethylamine benzoate. Additionally, 1.0, 2.5, 5.0, 10, 20 and 30 wt% of ZM was added to the various experimental resins. The DC was accessed by Fourier Transform infrared spectroscopy. For cytotoxicity, immortalized mouse fibroblasts were exposed to the experimental resin extracts. An MTT assay was used to access the cytotoxicity. The AA against *Streptococcus mutans* UA159 was accessed by the agar diffusion method. An improvement in the DC in all concentrations of ZM was observed. The greater the amount of ZM on the experimental resin, the less the cytotoxicity was provoked. Three ZM concentrations showed AA that exhibited various inhibition growth zones with 10, 20 (10 mm) and 30 wt% (15 mm).

1 Introduction

One ultimate goal of restorative dentistry is to produce dental restorations that replace the lost tooth structures with materials that have physical, mechanical and biological properties similar to organic dental tissues. There has been an increased application of resin-based materials in restorative dentistry, and dental adhesive systems should be used to promote the union between resin-based materials and the remaining tooth structure [1, 2]. Contemporary dental adhesives have exhibited satisfactory performance in short-term investigations [1, 2]. However, the combined degradation of adhesive polymer and collagen has been considered the main cause of dental adhesion degradation and consequent restoration loss [2–5]. Therefore, many researchers have proposed strategies to improve the adhesive's polymer quality [6–8]. Recently, a new strategy was used to increase the longevity of the adhesive restorations, which was the inhibition of matrix metalloproteinases (MMPs) present in the dentin collagen [9–11]. This strategy consists of the application of chlorhexidine on the collagen fibrils that become exposed after acid conditioning [9–11]. MMPs form a family of metal-dependent proteolytic enzymes that, collectively, are capable of degrading all kinds of extracellular matrix protein components, such as collagen. Some studies have also shown MMP's inhibitory effect on zinc-based dental materials, such as zinc oxide cements and dental amalgams [12–15]. Quite recently, was demonstrated that a zinc-based methacrylate showed a potential to inhibit the matrix metalloproteinase 2 [16].

Furthermore, one method to prevent enamel demineralization is to use an adhesive restorative material that is resistant to bacterial accumulation. Since secondary caries have been reported as the main reason for restoration failure in clinical trials [17], attention has been paid to the therapeutic effects revealed by direct filling materials [18–20]. With this purpose, several antimicrobial agents have been incorporated into dental products and were approved

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for intraoral use [19, 21, 22]. Fluoride and chlorhexidine are the most common preventive additives for oral use [18, 19]. Remineralization of dental structures has been demonstrated after fluoride usage, but the antibacterial effect is another important property because the inactivation of bacteria implies a direct strategy for preventing the cause of dental caries [19, 22]. Even though they initially exhibit ample strength, the amount of fluoride and chlorhexidine released are not long lasting [19, 22]. Therefore, new attempts to achieve a filling material with antibacterial effects are relevant to the inhibition of plaque accumulation on material surfaces and over dental structures around the restoration.

In this sense, zinc methacrylate is a monomer that contains a functional methacrylate group in its structure that is also found in other monomers present in the adhesive system, like TEGDMA, HEMA, and BISGMA. Thus, it is possible that MZ will copolymerize within a material based on methacrylate monomers, like most dental adhesive resin materials. The use of a copolymerizable monomer with zinc in its constitution is very promising. Zinc is a metallic chemical element with a strong antibacterial action [23]. Another interesting property of zinc is its potential effect as a MMPs inhibitor [12–15]. However, until now, no researchers have made any evaluation regarding the degree of conversion, cytotoxicity and antimicrobial activity of methacrylate-based material with zinc inclusion in their composition.

Thus, the aim of this study was to evaluate the effect of a zinc methacrylate addition in an experimental dental resin adhesive on the degree of conversion, cytotoxicity, and antimicrobial activity.

2 Materials and methods

2.1 Reagents

Triethyleneglycol dimethacrylate (TEGDMA) and camphoroquinone (CQ) were obtained from Esstech, Inc. (Essington, PA, EUA). Ethyl 4-dimethylamine benzoate (EDAB) and zinc methacrylate (ZM) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2 Formulations

The experimental adhesive resin was prepared with 3.0 g of TEGDMA and a binary photo-initiator system containing 0.4% of CQ and 0.8% of EDAB. Different concentrations of ZM were added as follows: 1.0, 2.5, 5.0, 10, 20 and 30 wt%.

2.3 Degree of conversion by FTIR spectroscopy

The degree of conversion from the experimental materials were evaluated using Fourier Transform infrared spectroscopy (Prestige 21 spectrometer, Shimadzu, Columbia, MD, USA) equipped with an attenuated total reflectance device composed of a horizontal ZnSe crystal and a 45° mirror angle (PIKE Technologies, Madison, WI, USA). A support was coupled to the spectrometer, fixing the light curing unit and standardizing the distance between the fiber tip to the sample at 5.0 mm. The monitoring scan mode of the IR solution software (SHIMADZU, Columbia, MD, USA) was used with the Happ-Genzel apodization at a range of 1,800 and 1,600/cm, at resolution of 8/cm and a mirror speed of 2.8 mm/s. The analysis was performed in a controlled room temperature of 23°C with the relative humidity <60%. The sample (3 μl) was directly dispensed by the ZnSe crystal and was scanned after the photo-activation, which was performed with a LED Raddi (SDI, Victoria, Australia) light-curing unit for 40 s, when samples were again scanned. The degree of conversion was calculated as previously described [8], considering the intensity of the carbon–carbon double bond stretching vibration (peak height) at 1,635/cm and using, as an internal standard, the carbon–oxygen double bond 1,710/cm from the polymerized and unpolymerized samples.

2.4 Cytotoxicity assay (MTT assay)

The cell culture medium was Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml). Mouse fibroblasts of the 3T3/NIH immortalized cell line were maintained as a stock culture in DMEM and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air until subconfluency.

The experimental adhesive resin was poured into sterile circular Teflon molds (5.0 mm diameter and 1.0 mm depth), covered with a Mylar strip, and photo-activated for 20 s at room temperature with a LED Raddi light-activation unit. The irradiance value was confirmed with a power meter (Demetron Research Corporation, OR, USA) with $\leq$700 mW/cm^2. The groups were divided according to the addition of zinc methacrylate on the experimental resin as previously described. Only one operator prepared the five specimens for each group.

Specimens were preincubated in a cell culture medium at one specimen per 1 ml of DMEM at 37°C and pH 7.2 for 24 h under a static condition. The extracts were filtered through 0.22 mm cellulose acetate filters (Millipore) and used for cytotoxicity experiments. Control groups involved

the use of polyethylene plastics as negative controls and non-cured dental adhesive model resin as positive controls.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell metabolic function according to mitochondrial dehydrogenase activity. Mouse fibroblasts 3T3/NIH (2×10^4/well) were maintained in DMEM in 96 well plates for 24 h. DMEM was removed and replaced with 200 µl of extracts from different groups with 10% of FBS. Cytotoxicity produced by different extracts was assessed at 24 h cell exposure time. After removing the extracts, cells were washed with phosphate-buffered saline (PBS) and then 200 µl of medium in 20 µl of MTT solution (5 mg of MTT/ml DMEM) were added to each well. After 5 h of incubation at 37°C in darkness, the blue formazan precipitate was extracted from the mitochondria using 200 µl/well dimethyl sulfoxide (DMSO) on a shaker for 5 min at 150 rpm. The absorption at 540 nm was determined spectrophotometrically.

2.5 Antimicrobial activity (agar diffusion assay)

The in vitro antimicrobial activity of the experimental dental resin adhesive containing zinc methacrylate was tested against the oral streptococci: *Streptococcus mutans* UA159.

The disk diffusion method was employed to determine the antimicrobial activity of the experimental dental resin adhesive containing different concentrations of zinc methacrylate. Briefly, a suspension of the tested microorganism (0.1 ml of 10^8 cells per ml) was spread on the solid media plates. The microorganism was seeded by pour plate. The *mutans* streptococci that were isolated for 18–24 h and grown on a brain–heart infusion agar were suspended in sterile brain–heart infusion broth. The suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale. A 400 µl portion of the test suspension was mixed with 40 ml of the brain–heart infusion agar at 45°C and poured onto a previously set layer of Mueller-Hinton agar. Nutritive media were prepared according to the manufacturer's instructions. All agar plates were prepared in 90 mm Petri dishes with 22 ml of agar giving a final depth of 4 mm. The inoculum procedure was appropriate for providing a semi-confluent growth of the microorganism tested. Six sterilized samples of the experimental dental resin with concentrations of zinc methacrylate (as previously described) were placed onto the inoculated agar plate. A sample of the resin monomer without zinc methacrylate was used as a control. Afterwards, the plates were incubated at 37°C under microaerophilic conditions (5–10% CO_2) and for an appropriate period of time (24–48 h).

Zones of inhibition of microbial growth around the samples were measured and recorded after the incubation time. The diameters of the inhibition zones were measured in millimeters using a Paquimeter. Three replicates were made and the experiment was repeated twice.

2.6 Statistical analysis

The degree of conversion, cell viability and antimicrobial activity data, were analyzed using the SigmaStat 3.5 (Systat Software Inc., San Jose, USA) statistical analysis software. The statistical analysis was performed by one-way ANOVA followed by a multiple-comparison Tukey post hoc test. The level of significance was set at $P < 0.05$. To evaluate the correlation between the degree of conversion and cell viability a Pearson product-moment correlation coefficient was conducted.

3 Results

The addition of zinc methacrylate (ZM) significantly influenced the degree of conversion even when only a small concentration (1 wt%) was added (Fig. 1). No significant difference was observed in the degree of conversion when ZM was added in concentrations between 1 and 30 wt% (Fig. 1).

The results for the mitochondrial reducing activity after cell contact with the extracts for 24 h are presented in Fig. 2. A decrease in cell viability after exposing the mouse fibroblasts 3T3/NIH to the extracts was observed in all tested groups in relation to the polyethylene plastic's

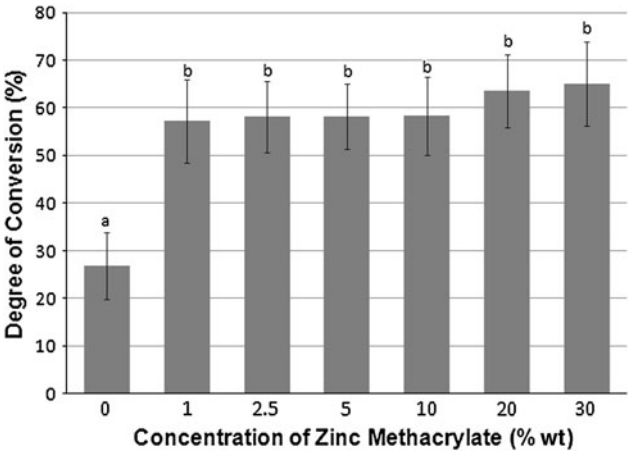

Fig. 1 Cytotoxicity provoked by eluates (24 h) from dental adhesive model resin added with different concentrations of zinc methacrylate on mouse fibroblasts 3T3/NIH. Percentage of absorbance from each group compared with control was calculated. *Different letters* denotes significant difference ($P < 0.05$) when compared with negative control (polyethylene plastics). Positive control (non-cured dental adhesive model resin) was the most cytotoxicity group, showing a cell viability of 0%

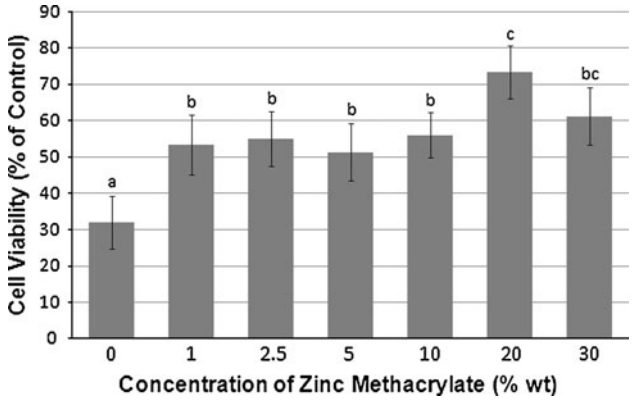

Fig. 2 Effect of the addition of zinc methacrylate (ZM) on the degree of conversion (DC) of the experimental dental resin adhesive photo-activated for 20 s. Note that the addition of ZM showed a significant increase on the DC. The influence of 1% of ZM was almost the same of 30% of ZM. *Different letters* denotes significant difference ($P < 0.05$)

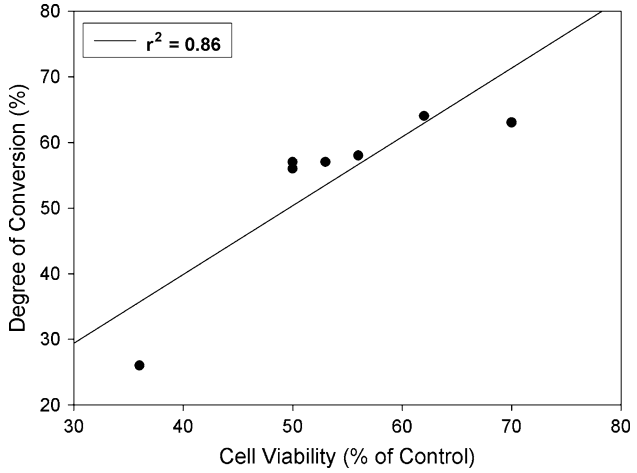

Fig. 3 Pearson product-moment correlation coefficient between degree of conversion and cell viability. A strong relationship between degree of conversion and cell viability was observed according to results obtained after the addition of the zinc methacrylate on the experimental dental resin adhesive ($r^2 = 0.86$)

nontoxic control group (Fig. 2). On the other hand, the non-cured dental adhesive model resin was the most cytotoxic group, showing a cell viability of 0 wt%. The addition of ZM resulted in a continuous reduction in cytotoxicity when compared with the control group (Fig. 3). As seen in Fig. 3, a Pearson correlation was conducted, and it showed that the degree of conversion and cell viability were positively correlated ($r^2 = 0.86$).

Regarding the antimicrobial activity, the samples of the experimental adhesive presented a zone of growth inhibition at concentrations of 10, 20 and 30 wt% of ZM. The lattermost demonstrated the highest antimicrobial activity (Fig. 4). The experimental resin with 10 and 20 wt% of ZM showed a zone of inhibition of microbial growth

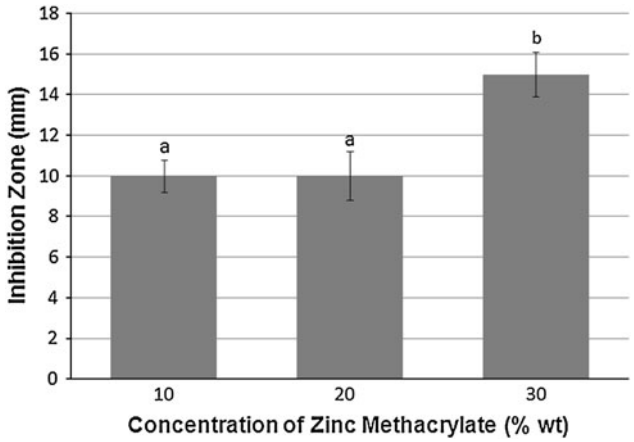

Fig. 4 Antibacterial effect of zinc methacrylate-incorporated adhesive system against *Streptococcus mutans* UA159 determined by the agar diffusion test ($n = 9$). *Different letters* denotes significant difference ($P < 0.05$)

around the samples of 10 mm, while experimental resin with 30 wt% of ZM showed 15 mm, which was statistically significant difference as shown in Fig. 4.

4 Discussion

The addition of zinc methacrylate (ZM) significantly altered all properties evaluated in this study. Our results showed that ZM significantly improved the degree of conversion of the experimental resin formulated. Even the addition of only 1 wt% of ZM was able to significantly increase the degree of conversion of the experimental resin compared to the group without ZM addition (Fig. 1). On the other hand, antimicrobial activity was observed only with the addition of larger amounts of ZM (10, 20, and 30 wt%). ZM addition also significantly influenced the cytotoxicity observed (Fig. 2), as did the degree of conversion (Fig. 3).

However, these data must be considered with care, since other adhesive formulations can be proposed that would produce different results. The degree of conversion is directly related to the mechanical properties of a given polymer: when a larger polymer conversion occurs, a higher degree of conversion occurs, and better mechanical properties are observed [24]. Our results also showed that the degree of conversion influenced the cytotoxicity level (Fig. 3). A hypothetical explanation for this is that ZM may have increased the reticulation between the methacrylate co-monomers, thus decreasing the TEGDMA output of the system. TEGDMA is usually used as a diluent monomer due to its characteristically low viscosity. Several studies have shown that TEGDMA can be leached out of the dental resinous materials and cause cytotoxicity [24–26]. So, ZM may be a promising monomer for the development of resinous materials.

Divalent zinc ions can effectively inhibit MMP proteolytic activity [12, 15]. Zinc-containing dental materials, such as zinc oxide-eugenol cements, zinc-containing amalgams, and zinc phosphate, have also demonstrated a capacity to inhibit metalloproteinase two and nine [13, 14]. The mechanism of enzyme inactivation by metals is not completely understood. It is assumed that metal ions bind with amino acid residues, causing conformational changes that inactivate the catalytic function of enzymes. Previous study has shown that the mechanism of zinc inhibition of carboxypeptidase A, a zinc metalloproteinase, occurs due to the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active site of the enzyme [27]. The non-competitive inhibition by other heavy metal ions is attributed to binding of the ion to a site distinct from the active site [27]. Recently was demonstrated that ZM has an important potential of matrix metalloproteinase two inhibition [16]. The possibility of using a copolymerizable matrix metalloproteinase inhibitor with the other methacrylate monomers is highly promising.

Many studies have been conducted to produce and identify dental materials with bactericidal activity [19, 21, 23]. The combination of antibacterial agents in dental resinous materials can ensure greater stability of antimicrobial activity. Zinc is an important antibacterial agent, and a methacrylate monomer with zinc in its constitution could be a promising component for resin-based material formulations like dental adhesives, sealants, temporary resin-based cements and resin modified glass ionomer cements.

The present findings support the evidences that the presence of zinc on the methacrylate monomer produced an antimicrobial activity, with a 30 wt% (ZM) concentration being the most effective, followed by the concentrations of 20 and 10 wt%. The other samples of the experimental adhesive presented no zone of growth inhibition. The control (the resin monomer without zinc methacrylate) also did not form any inhibitory zone against the strain of *S. mutans* tested. The diffusion methods are the most often employed in research even though they present some shortcomings. In some cases, the diffusion techniques can be used for antimicrobial screening but they can never be used as a definitive method. So, more in-depth studies are required to investigate the anti-microbial effect of ZM over other microorganisms with other methodologies, such as the biofilm assays.

5 Conclusion

Collectively, the results of our study demonstrate that the addition of ZM to our experimental formulation of dental adhesive resin produced an improved degree of conversion, reduced cytotoxicity, and produced an antibacterial effect in the highest concentration. However, additional studies are needed to confirm the promising results observed in the present studies.

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