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Cytotoxicity and effect on protease activity of copolymer extracts containing catechin

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ABSTRACT

Objective: To evaluate cytotoxicity and effect on protease activity of epigallocatechin-gallate extracted from experimental restorative dental copolymers in comparison to the control compound chlorhexidine.
Methods: Copolymer disks were prepared from bis-GMA/TEGDMA (70/30 mol%) containing no compound (control) or 1% w/w of either epigallocatechin-gallate or chlorhexidine. MDPC-23 odontoblast-like cells were seeded with the copolymer extracts leached out into deionized water. Cell metabolic activity was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24, 48, 72 h. Inhibition of protease activity by resin extracts was measured by a collagenolytic/genatinolytic enzyme activity assay and gelatin zymography. Data for MTT and protease inhibition were analyzed using two-way ANOVA followed by Tukey or Bonferroni *post hoc* tests ($\alpha = 0.05$).
Results: The MTT revealed that at 72 h, extracts from control (16.7%) and chlorhexidine (22.3%) copolymers induced significant reduction in cell metabolism ($p < 0.05$). All copolymer extracts caused enzymatic inhibition in a dose dependent manner ($p < 0.01$). Even when highly diluted, epigallocatechin-gallate extract had a significant antiproteolytic activity ($p < 0.05$). Zymograms showed that all extracts reduced activity of MMP-2 and MMP-9 (pro- and active forms), with MMP-9 exhibiting the highest percentage inhibition revealed by densitometry.
Conclusions: Epigallocatechin-gallate and chlorhexidine extracts did not exert cytotoxicity on evaluated cells when compared to control extracts. Both compounds retained antiproteolytic activity after extraction from a dental copolymer.
Clinical significance: Once extracted from a dental copolymer, epigallocatechin-gallate is not cytotoxic and retains antiproteolytic activity. These results may allow incorporation of epigallocatechin-gallate as a natural-safe alternative to chlorhexidine in functionalized restorative materials.

1. Introduction

Resin composites are used in a variety of applications in dentistry including restorations, liners, cores, cementation, and endodontic sealers. The composition of resins has evolved significantly since they were introduced, first aiming to improve wear resistance and polish, lower the polymerization shrinkage and later to develop self-adhesive materials (Chen, 2010). However, secondary caries (Rasines Alcaraz et al., 2014) and loss of bond strength due to proteolytic activity of collagenolytic and gelatinolytic enzymes in the hybrid layer (Tjäderhane et al., 2013), still remain to be the problems reducing the composite filling longevity. Enzymes such as MMP-2 and MMP-9, localized in dentin or saliva, may have a fundamental role in dentin organic matrix degradation and caries progression (Tjaderhane et al., 1998; Mazzoni et al., 2007). Both MMP-2 and MMP-9 were indeed found to be among the most abundant proteases found in caries-affected dentin (Vidal et al., 2014). Future developments should

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thus aim to produce functionalized materials, with therapeutic activities, such as antibacterial and/or antiproteolytic properties (Tjäderhane et al., 2013; Jandt & Sigusch, 2009; Delaviz, Finer, & Santerre, 2014).

Chlorhexidine is capable of arresting caries when applied to dentin (Van Rijkom, Truin, & vant Hof, 1996). Chlorhexidine also inhibits dentin endogenous proteases such as MMPs and cysteine cathepsins (Tjäderhane et al., 2013). This antiproteolytic effect prevents hybrid layer degradation and increases dentin bond strength durability (Tjäderhane et al., 2013), and may also participate in the prevention of dentinal caries progression (Garcia et al., 2009). The release of chlorhexidine from resins by Fickian diffusion has been widely tested in terms of mechanical and biological properties (Leung et al., 2005; Anusavice, Zhang, & Shen, 2006; Pallan, Furtado Araujo, Cilli, & Prakki, 2012), confirming the antibacterial efficacy also when incorporated into adhesive resin (Hiraishi, Yiu, King, Tay, & Pashley, 2008). However, certain drawbacks of this compound such as its synthetic nature and a possible cytotoxicity to dentin odontoblastic cells (Lessa, Nogueira, Huck, Hebling, & Costa, 2010; de Souza, de Aquino, de Souza, Hebling, & Costa, 2007) motivate researchers to investigate alternatives.

Epigallocatechin-gallate (EGCg), a natural polyphenol from green tea, has been shown to be effective antimicrobial against *Streptococcus mutans* and in inhibiting acid production in dental biofilm (Hirasawa, Takada, & Otake, 2006; Xu, Zhou, & Wu, 2012). Incorporation of EGCg has also been shown not to adversely affect the mechanical properties of different copolymer compositions (Pallan et al., 2012), and EGCg released from these copolymers retains antibacterial activity against *S. mutans* (Mankovskaia, Levesque, & Prakki, 2013). Moreover, it has been reported that EGCg inhibits dentinal proteases (Kato et al., 2012), thus preserving the long-term dentin bond strength with an effect equal to chlorhexidine (Santiago, Osorio, Neri, Carvalho, & Toledano, 2013). However, the protease activity of compounds released from dental copolymers has not yet been demonstrated. It was therefore the objective of this study to investigate the cytotoxicity and protease-inhibiting activities of EGCg, released by an experimental dental copolymer, and compare that to chlorhexidine. The working null hypotheses were (1) extracts from EGCg and chlorhexidine incorporated copolymers will not exert cytotoxicity against MDPC-23 odontoblast-like cells, and (2) extracts from EGCg and chlorhexidine incorporated copolymers will not exhibit collagenolytic and gelatinolytic activities.

2. Materials and methods

Chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.1. Formulation of copolymers and compound extraction

Experimental copolymer disks (7 mm diameter × 2 mm high) were prepared from bis-GMA (bisphenol glycidyl dimethacrylate) and TEGDMA (triethyleneglycol dimethacrylate) at 70/30 mol% ratio, containing no compound (control) or with 1% w/w of either epigallocatechin-gallate or chlorhexidine diacetate. The 1% compound ratio was chosen based on previous study that reported stability in mechanical properties of same copolymer incorporated with 1% of either epigallocatechin-gallate or chlorhexidine diacetate (Pallan et al., 2012). Resins were activated for visible light polymerization (Demi LED, Kerr Co., Middleton, USA; 540 mW/cm^2) by the addition of camphorquinone and 2-(dimethylamino) ethyl methacrylate (0.2% w/w each) (Pallan et al., 2012), and stored at 6 °C until use.

A UV–vis spectrophotometer (Synergy HT Multi-Mode Microplate Reader, BioTEK, Winooski, VT, USA) was used to analyze the compound release rates ($n = 3$) after 24 h storage in 1 mL deionized water (EGCg OD_{297} and chlorhexidine OD_{257}) at 37 °C. For the following tests, the copolymer extracts were concentrated 10× in volume (Eppendorf Concentrator Plus, Eppendorf AG, Hamburg, Germany).

2.2. MDPC-23 odontoblast-like cell culture

The immortalized mouse dental papilla MDPC-23 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; with high glucose, L-glutamine and sodium pyruvate) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), with 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL fungizone (Gibco). The cells were sub-cultured every 3 days and allowed to grow in a humidified incubator at 37 °C with 5% CO_2 (Isotemp; Fisher Scientific, Pittsburgh, PA, USA). Studies have shown that MDPC-23 synthesizes dentin-specific proteins (*i.e.*, dentin sialoprotein) that are synthesized mainly by odontoblasts. These findings support the idea that MDPC-23 may serve as a valuable *in vitro* model for studies of functional roles of odontoblasts (Shin, Yeon, Oh, & Kim, 2006).

2.3. MTT cytotoxicity assay

MDPC-23 cells were seeded in DMEM in 96-well plate at 3 × 103 cell/200 μL/well and allowed to grow for 72 h. At day 3, the new medium was incorporated with the copolymer extracts diluted at 40 μL/100 μL. The cells were continuously cultured and evaluated at 24, 48 and 72 h cell growth time points ($n = 3$). The extract from control copolymer was used as positive control and PBS (Phosphate Buffered Saline) as negative control. Cells metabolic activity was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche Applied Science, Indianapolis, IN, USA) as previously described (Aranha, Giro, Hebling, Lessa, & Costa, 2010). Data were analyzed by two-way ANOVA and Tukey's *post hoc* test ($\alpha = 0.05$).

2.4. Gelatinolytic/collagenolytic activity assay

The effect of copolymer extracts on functional enzyme activity was assayed using EnzChek gelatinolytic/collagenolytic assay kit (Molecular Probes; Eugene, OR, USA) supplemented with type IV *Clostridium histolyticum* collagenase (Molecular Probes). The fluorescence of the collagen substrate is internally quenched and is only released when it is cleaved enzymatically into highly fluorescent, low molecular weight peptides. Different dilutions of copolymer extracts (80 μL to 1 μL) were mixed with quenched fluorescent substrates in a final volume of 200 μL of reaction buffer in 96-well plates ($n = 6$). The rate of proteolysis was determined by a Synergy™ Mx Monochromator-Based Multi-Mode Fluorometer (BioTek), operated at an absorption maxima of 495 nm and fluorescent emission maxima of 515 nm. The assay included a series of control collagenase standards as well as reagent blanks. The change in enzymatic activity by EGCg or chlorhexidine extracts was calculated (%) in comparison to the control extracts within each tested dilution. Data were analyzed by two-way ANOVA and Bonferroni's *post hoc* test ($\alpha = 0.05$).

2.5. Zymographic analysis

MMP-2 and MMP-9 were in-house gelatin-Sepharose column-purified from conditioned cell culture media as previously described (Mäkelä, Salo, Uitto, & Larjava, 1994). Aliquots of purified human MMP-2 (9 μL + 3 μL deionized water) or MMP-9 (12 μL)