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The preventive effect of grape seed extract on artificial enamel caries progression in a microbial biofilm-induced caries model

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ABSTRACT

Objectives: The aim of this study was to evaluate the effect of grape seed extract (GSE) on enamel caries lesion formation in an *in vitro Streptococcus mutans* biofilm model.
Methods: Enamel fragments were prepared from bovine incisors and divided into six treatment groups ($n = 12$): inoculated Brain Heart Infusion with 1% sucrose (BHIS), 1 mg/mL GSE, 2 mg/mL GSE, 3 mg/mL GSE, 10 ppm fluoride as NaF, and uninoculated BHIS. For biofilm formation, tooth fragments were incubated anaerobically in polystyrene 6-well tissue culture plates containing BHIS, the respective agents, and *S. mutans* (1×10^5 CFU/mL) for 24 h at 37 °C. Culture medium was replaced with fresh BHIS and respective agents daily over a 7-day period. Following caries lesion formation, lesion depth (LD) and relative optical density (ROD) were determined by polarized light microscopy (PLM) and confocal laser scanning microscopy (CLSM), respectively, to evaluate lesion progression.
Results: LDs of the 2 mg/mL GSE group (122.86 ± 13.41 μm) and the 3 mg/mL GSE group (111.92 ± 11.39 μm) were significantly smaller than those of the 1 mg/mL GSE (198.33 ± 17.70 μm) and control groups (210.86 ± 15.50 μm) ($p < 0.05$). Compared with the 2 mg/mL and 3 mg/mL groups, the control and 1 mg/mL GSE groups showed significantly lower ROD values when depth was less than 200 μm, indicating greater mineral loss.
Conclusions: Dose-dependent GSE inhibits *in vitro* enamel caries formation due to its ability to suppress growth of *S. mutans* and the formation of biofilm and thus may be a promising Q4 agent for enamel caries prevention.

1. Introduction

Although current preventive measures—including the administration of fluoride and broad-spectrum antimicrobials, along with the reduction of sucrose intake and effective oral hygiene habits—have been demonstrated to decrease caries prevalence,[1] dental caries remains one of the most prevalent diseases in humans.[2] Dental caries is initiated by demineralization of the tooth surface through acid production from sugar

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by plaque biofilm. *Streptococcus mutans* is considered the principal cariogenic pathogen and plays a decisive role in the development of dental caries.[3–5] Various measures have been developed to counteract the onset or progression of dental caries. One preventive method is the use of antimicrobial agents, such as chlorhexidine or NaF, to limit the growth and biofilm formation of cariogenic microorganisms, especially *S. mutans*, in the oral cavity.[3–6] However, the conventional chemical antimicrobial agents have demonstrated some limitations and have not been recommended for regular caries prevention.[7] In recent years, much attention has been focused on plant-derived natural antimicrobial compounds, with potential use as alternatives to the common chemicals used for caries prevention.[8–17]

Polyphenolic compounds (polyphenols) are secondary metabolites of plants and are commonly found in both edible and non-edible plants or plant-based foods and beverages. Their health benefits (*e.g.*, antioxidant, anticancer, and anti-inflammatory) have been emphasized within the last decade.[18] The consumption of polyphenol-rich foods or beverages has also been reported to benefit oral health, with antigingivitis and anticaries properties.[14,18–22] In recent years, the antimicrobial and antiplaque activity of plant polyphenols has been demonstrated in many *in vitro* studies.[16,17,23–25] Grape seed extract (GSE), derived from the seeds of *Vitis vinifera*, is rich in polyphenolic compounds. It consists mainly of free monomeric flavanols, *i.e.*, the proanthocyanidins (PACs), as well as their dimeric, trimeric, tetrameric, and higher oligomeric forms, termed the oligomeric proanthocyanidins (OPACs). Examples of PACs contained in GSE are catechin, epicatechin, and epicatechin-3-O-gallate. These monomers are the structural building blocks of the OPCAs contained in GSE. GSE has attracted much attention in recent years due to its well-documented antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic properties.[26,27] PACs have remarkable dentine-specific protective effects by decreasing biodegradation rates and enhancing the mechanical properties of the organic matrix.[28] The antimicrobial activity of GSE has been reported in the literature, but information on its effect on the growth of cariogenic bacteria, especially their resistant biofilm, is limited.[29,30]

Using an *in vitro* pH-cycling model, researchers have demonstrated the positive effect of GSE on artificial root caries by stabilizing the collagen-based tissues and promoting remineralization.[31,32] However, there are no available data on the anticariogenic potential of GSE (in terms of its antibacterial and antibiofilm properties) in combating microbial biofilm-induced artificial enamel caries. A more clinically relevant model is needed.[33] It is hypothesized that GSE inhibits the growth and biofilm formation of *S. mutans*, thereby preventing the progression of artificial enamel caries. This study evaluated the preventive effect of GSE on caries lesion formation in an *in vitro S. mutans* biofilm model.

2. Materials and methods

2.1. Test bacteria and grape seed extract (GSE)

The test bacterium used in this study was *S. mutans* UA159. Cultures were routinely grown in Brain Heart Infusion broth (BHI; Difco, Detroit, MI, USA) under anaerobic conditions (Forma anaerobic chamber, 80% N_2, 10% CO_2, and 10% H_2) for 24 h at 37 °C overnight. For *S. mutans* biofilm formation, BHI with 1% sucrose (BHIS) was used. The grape seed extract (GSE) obtained from the seeds of *Vitis vinifera* was purchased from MegaNatural, Polyphenolics (Madera, CA, USA). It consists of 97.8% proanthocyanidins (PA) according to data provided by the manufacturer. For assays, GSE was dissolved in distilled water and filtered through an MF-Millipore membrane (0.22 μm, Carrigtwohill Co., Cork, Ireland).

2.2. Determination of minimum inhibitory concentration (MIC) and minimum biofilm inhibition concentration (MBIC)

The MIC and MBIC of GSE of *S. mutans* were determined by a microdilution method in 96-well microliter plates (Corning, NY, USA). Each well contained *S. mutans* (final concentration 1×10^5 CFU/mL), BHI/BHIS medium, and serially diluted GSE (0.125–16 mg/mL). Control wells contained BHI/BHIS without GSE, uninoculated BHI/BHIS, or GSE alone. Chlorhexidine digluconate solution (CHX, Sigma, St. Louis, MO, USA) was used as a positive control. All plates were incubated in an anaerobic chamber (Forma anaerobic chamber, 80% N_2, 10% CO_2, and 10% H_2) at 37 °C for 24 h, after which growth was determined spectophotometrically at 550 nm by means of a microplate reader (PowerWave 200, Bio-Tek Instruments, Winooski, VT, USA). The MIC was defined as the lowest concentration of GSE that inhibited the visible growth of *S. mutans* ($OD_{550} < 0.05$). MBIC was defined as the lowest GSE concentration that inhibited formation of *S. mutans* biofilms.[17] The data were reported as the median of at least 3 independent tests.

2.3. Enamel specimen preparation

Seventy-two extracted sound bovine incisors were used. The crowns were cut from the roots at the cemento-enamel junction with a cylindrical diamond bur (No. 557D, Brasseler, Savannah, GA, USA) in a high-speed handpiece. One enamel disc fragment (5 mm × 4 mm × 3 mm) was obtained *per* crown by means of a low-speed diamond blade (Isomet 1000, Buehler, Lake Bluff, IL, USA). Subsequently, enamel surfaces were ground flat and polished on a rotating polishing machine under water cooling (Phoenix Alpha; Buehler, Düsseldorf, Germany) with progressively finer grades of SiC grinding paper. Approximately 150–200 μm of surface enamel was removed. In total, 72 fragments were obtained and sealed with acid-resistant nail polish (Revlon Corp., New York, NY, USA), except for a 3 mm × 2 mm window on each fragment.

2.4. In vitro caries lesion formation induced by S. mutans biofilm

Seventy-two bovine enamel fragments were randomly divided into six treatment groups ($n = 12$) as follows: Group 1, Brain Heart Infusion with 1% sucrose (BHIS, as control); Group 2, BHIS + 1 mg/mL GSE; Group 3, BHIS + 2 mg/mL GSE; Group 4, BHIS + 3 mg/mL GSE; Group 5, BHIS + 10 ppm

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