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Original Article

Effect of Brazilian green propolis on oral pathogens and human periodontal fibroblasts

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ABSTRACT

Objectives: Plaque biofilm is the main etiological factor in dental caries and periodontitis. This study aimed to evaluate the effects of Brazilian green propolis (BGP), which has antibacterial and anti-inflammatory properties, on the growth of oral pathogens, and its cytotoxicity to adult human gingival fibroblasts (HGFs) and periodontal ligament fibroblasts (HPDLFs).
Methods: *Streptococcus mutans*, *Streptococcus sanguinis*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* were used to evaluate the antibacterial action of BGP. Media containing different concentrations of BGP were prepared, and their effect on bacterial growth was determined by measuring their optical density. The cytotoxicity of BGP to HGFs and HPDLFs was determined by measuring the proliferation and viability of cells with the Alamar Blue assay.
Results: No significant growth of *P. gingivalis*, *S. mutans,* or *S. sanguinis* was observed when exposed to concentrations of BGP above 2000 μg/ml, but growth of *A. actinomycetemcomitans* was not affected at this concentration. Growth of *P. gingivalis and S. sanguinis* was significantly inhibited when exposed to BGP at a concentration of 100 μg/ml. Growth of *S. mutans* was significantly inhibited in medium containing BGP at 500 μg/ml. Cellular viability (LD50) of HGFs and HPDLFs was not observed at 2000 μg/ml BGP.
Conclusions: These results suggest that BGP has antibacterial properties but is not significantly cytotoxic to periodontal fibroblasts.

1. Introduction

Dental caries and periodontitis are major diseases of the oral cavity and are the principal causes of tooth loss. Dental plaque is a biofilm that adheres to teeth and promotes these diseases. Plaque is classified according to its location. Supragingival plaque is located around the tooth crown, and subgingival plaque, which is involved in periodontitis, extends into the gingival sulcus and periodontal pockets.

The bacterial flora in plaque depends on the site of plaque formation. *Streptococcus mutans* and *Lactobacillus* spp. inhabit the supragingival area. These pathogens are thought to cause dental caries, because they are detected at a high frequency at the site of dental caries, produce extracellular glycan and lactic acid, and are able to proliferate in acidic conditions [1–3]. Socransky et al. reported that a variety of pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia* are involved in periodontitis [4]. Detection of these bacterial species greatly increases as periodontitis advances, and many of these species are simultaneously detected in severe cases of periodontitis. Based on these findings, periodontitis is considered to be a complex infection involving multiple causative pathogens. Furthermore, these species of bacteria are used as indicators when treating periodontitis.

Mechanical removal of dental plaque and selective elimination of pathogenic bacterial species from plaque are important in the treatment and prevention of dental caries and periodontitis [5–10]. Chemical plaque control involves the use of mouthwashes that contain a bactericidal and/or antibacterial component, or the use of a local drug delivery system. However, mouthwashes do not have a direct effect on plaque but prevent recolonization of plaque [11–13]. Plaque recolonization occurs at a rapid rate after removal of plaque, and gingivitis can occur after accumulation of bacteria

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for 3 days [5]. In recent years, mechanical methods and drugs to remove plaque have been extensively investigated.

Propolis is a viscous substance that is collected by honeybees from plants and has been used by humans as a treatment for a variety of diseases. Propolis contains flavonoids and cinnamic acid derivatives, and has broad antibacterial and anti-inflammatory properties, as well as a wide range of physiological activities, including the inhibition of glucosyl transferase [14]. A decrease in the frequency of detection of intraoral *S. mutans* has been reported following the use of mouthwash containing propolis [15]. However, few studies have examined the inhibitory effect of Brazilian green propolis (BGP) on the growth of oral bacteria or its periodontal cytotoxicity. Therefore, we examined the effects of BGP on the growth of oral bacteria and its cytotoxicity to human gingival fibroblasts (HGFs) and periodontal ligament fibroblasts (HPDLFs).

2. Materials and methods

2.1. Bacterial species and preculture conditions

The following four species were used in this study: *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 49296), *A. actinomycetemcomitans* (ATCC 29522), and *P. gingivalis* (W83, ATCC 33277). *S. mutans* was cultured in brain heart infusion (BHI) medium (Becton Dickinson and Company, Franklin Lakes, NJ) under aerobic conditions at 37 °C in an incubator. *S. sanguinis* and *A. actinomycetemcomitans* were cultured in BHI medium at 37 °C in a CO_2 gas incubator (5% CO_2, 95% air). *P. gingivalis* was cultured in BHI medium containing 4 μg/ml hemin (Acros Organics, Kanagawa, Japan) and 0.4 μg/ml menadione (Sigma-Aldrich, St. Louis, MO) (BHI/HM) at 37 °C in an anaerobic incubation chamber (Hirasawa Company, Tokyo, Japan) (N_2: 80%, CO_2: 10%, H_2: 10%).

2.2. Preparation of propolis

BGP was extracted with a mixed solvent containing water and ethanol (kind gift from Nihon Natural Therapy Co. Ltd., Tokyo, Japan). BGP was serially diluted with ethanol (Wako, Osaka, Japan) before being added at the appropriate concentration to the media. The final concentration of ethanol in the culture medium was less than 0.1%.

2.3. Measuring inhibition of bacteria

Liquid media containing bacteria were prepared to a total volume of 3 ml. BGP was added to yield final concentrations of 50, 100, 200, 400, 500, 100, and 2000 μg/ml. Only ethanol was added to the control groups. The precultured bacteria were collected and washed three times with 10 mM phosphate buffer saline (Dulbecco's PBS [-], Nissui Pharmaceutical, Tokyo, Japan, pH 7.4) to obtain a bacterial suspension. The bacteria in 10 mM Dulbecco's PBS (Nissui Pharmaceutical, pH 7.4) were diluted 25-fold with BGP-containing medium. *S. mutans* was cultivated in an incubator at 37 °C. *S. sanguinis* and *A. actinomycetemcomitans* were cultured at 37 °C in a CO_2 gas incubator, and *P. gingivalis* was cultured at 37 °C in an anaerobic incubation chamber. The growth of bacteria was monitored by measuring the optical density at 660 nm using a Miniphoto 518R spectrophotometer (Taitec Co., Tokyo, Japan). The data were collected in duplicate, and at least three independent experiments were performed ($n=6$).

2.4. Isolation and culture of HGFs and HPDLFs

Approval for this study was obtained from the Ethical Review Committee of the Nippon Dental University School of Life Dentistry at Niigata (approval number: ECNG-H-162). Oral informed consent was obtained from all patients. HGFs and HPDLFs were obtained during tooth extraction (patients: 6 females; aged 15–25 years; mean age: 21.7 years) at Nippon Dental University Niigata Hospital. Normal HGFs and HPDLFs were obtained from a healthy patient with non-inflamed gingiva and periodontal ligament explants according to the protocol described by Giannopoulou [16]. HGFs and HPDLFs were cultured in Dulbecco's Modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco Life Technologies, Carlsbad, CA) with 15% fetal bovine serum (FBS) (JR Scientific, Woodland, CA), antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) (Gibco Life Technologies), and antimycotics (250 μg/ml amphotericin B) (Gibco Life Technologies). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C until outgrowth appeared. The cells were cultured in a monolayer to the first passage and then treated with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid for 5 min, collected by centrifugation, washed, and suspended in PBS containing 1% FBS. The HGFs and HPDLFs were regulated at 3×10^3 cells/100 μl and were pipetted into a 96-well microplate.

2.5. Evaluation of cellular viability

HGFs and HPDLFs were exposed to media and BGP at various concentrations (50–2000 μg/ml) for 24 h after preculture (5% CO_2, 95% air, 37 °C, for 24 h) on a 96-well microplate. The control group (0 μg/ml BGP) was exposed to media and ethanol for 24 h after preculture. Cellular viability was measured by the resazurin reduction assay using Alamar Blue (Invitrogen, Carlsbad, CA) and a fluorescent plate reader (Fluoroskan Ascent FL, Dainippon Seiyaku, Osaka, Japan) at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. The data were collected in duplicate, and at least three independent experiments were performed ($n=6$).

2.6. Statistical analysis

Statistical analysis was performed using Social Sciences 13.0J for Windows statistical analysis software (SPSS Japan, Tokyo, Japan). Data were analyzed by one-way analysis of variance, followed by Tukey's test. The level of significance was set to $p < 0.01$.

3. Results

Growth curves for the different species of bacteria are shown in Figs. 1 and 2. The inhibitory concentrations of BGP differed depending on the species of bacteria. Growth of *S. mutans* was significantly inhibited at concentrations of 500 μg/ml when compared with the control ($p < 0.01$, Fig. 1a). This inhibition was dose-dependent. Complete inhibition occurred at 2000 μg/ml ($p < 0.01$, Fig. 1a). Growth of *S. sanguinis* was significantly inhibited at concentrations of 50 μg/ml when compared with the control ($p < 0.01$, Fig. 1b). This inhibition was dose-dependent. Complete inhibition occurred at 200 μg/ml ($p < 0.01$, Fig. 1b).

The inhibitory effect of BGP on *A. actinomycetemcomitans* was weak. Growth of bacteria was observed even at the highest dose of 2000 μg/ml (data not shown). The inhibitory effect on the growth of *P. gingivalis* depended on the species. Significant inhibition of growth was observed for the W83 strain at BGP concentrations of 50 μg/ml when compared with the control ($p < 0.01$, Fig. 2a). No growth of W83 was observed at a concentration of 200 μg/ml ($p < 0.01$, Fig. 2a). Growth of ATCC 33277 was significantly inhibited at BGP concentrations of 100 μg/ml ($p < 0.01$, Fig. 2b) and greater. No growth of ATCC 33277 was observed at a concentration of 400 μg/ml ($p < 0.01$, Fig. 2b).

Cell viability of HGFs in the control group was significantly different from that in samples exposed to concentrations of BGP