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Formulation and Evaluation of a Mucoadhesive Thermoresponsive System Containing Brazilian Green Propolis for the Treatment of Lesions Caused by Herpes Simplex Type I

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ABSTRACT

The aim of the present work was to develop a topical delivery system that contains Brazilian green propolis extract (PE-8) to increase efficiency and convenience when applied to herpetic lesions. The cytotoxicity and antiherpetic activity was determined *in vitro* and *in vivo*. The PE-8 was added to a system that contained poloxamer 407 and carbopol 934P. The *in vitro* characterization of the system included rheological studies, texture profile analysis, and mucoadhesion analysis. The PE-8 inhibited the virus during the phase of viral infection, induced virion damage, and exhibited an ability to protect cells from viral infection. The system had advantageous mucoadhesive properties, including a suitable gelation temperature of approximately 25°C for topical delivery, a desirable textural profile, and pseudoplastic behavior. The *in vitro* release study showed a rapid initial release of the PE-8 in the first 3 h, and the rate of drug release remained constant for up to 24 h. The system appeared to be macroscopically and microscopically innocuous to skin tissue. Therefore, the mucoadhesive thermoresponsive system that contained the PE-8 appears to be promising for increasing bioavailability and achieving prolonged release of the PE-8 when applied to skin lesions caused by herpes simplex virus type 1.

Introduction

The incidence of diseases that are caused by herpes simplex virus type 1 (HSV-1) has increased in recent years. HSV-1 causes a variety of infections in humans, including mucocutaneous infections, mainly orofacial lesions.[1] The current therapeutic standard for the management of HSV infection is based on nucleoside analogs.[2] However, the appearance of resistant viruses in immunocompromised patients continues to be problematic, which may result in such complications as encephalitis. Thus, new antiherpetic compounds need to be developed with different mechanisms of action. One group of potential therapeutic agents for the treatment of HSV is composed of substances of natural origin.[3]

Propolis is a natural bee product and it has a wide range of biological activity. The antiviral effects of propolis against various types of viruses, including HSV, have been reported.[4-6] Propolis that is produced in southeastern Brazil is known as green propolis and its main source is the plant *Baccharis dracunculifolia* (Asteraceae).[7] It possesses significant amounts of flavonoids and polyphenols, many of which are not present in European, North American, or Asian propolis.[8]

The application of formulations to the oral mucosa has certain limitations, including the rapid elimination of locally applied

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drugs.[9] One alternative to prolong the residual time of the drug at the application site is to use thermoresponsive and mucoadhesive platforms.[10-13]

These systems have a reversible state-transition property that enables a cool solution to flow on the skin and be distributed to the surface. However, as the temperature increases, the formulations exhibit increases in viscosity and retention time at the administration site for the desired period of time. They make intimate contacts to generate a nonocclusive gel at body temperature.[9] Moreover, bioadhesive polymers, such as carbopol 934P (C934P) and poloxamer 407 (P407), also prolong the retention time on oral mucosa and thus decrease the frequency of drug administration and increase patient compliance to therapy.[14]

In the present study, the *in vitro* anti-HSV-1 activity of Brazilian green propolis extract (PE) and efficacy against cutaneous HSV-1 infection in a mouse model were examined. To optimize the therapeutic action against HSV-1, we sought to develop an *in situ* gel formulation that is composed of C934P and P407 for local delivery of the PE to skin lesions in oral mucosal that are caused by HSV-1.

Materials and Methods

Chemicals

P407 and C934P were purchased from Sigma-Aldrich (St. Louis, MO) and B.F. Goodrich (Brecksville, OH), respectively. Triethanolamine was purchased from Galena (Campinas, São Paulo, Brazil). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Chicago, IL). and Penicillin, streptomycin, sulforhodamine B, acyclovir (ACV), and porcine stomach mucin type II were purchased from Sigma-Aldrich. Ketamine chloride (Ceva, Paulínia, Brazil), Permount™ (Fisher Scientific, Pittsburgh, PA), trichloroacetic acid (Synth, São Paulo, Brazil), and Zovirax™ (GlaxoSmithKline Brasil Ltd.) were also used.

Preparation and Characterization of PE

Three samples of Brazilian green propolis from hives of the bee *Apis mellifera* L., classified as type BRP[8,15] were purchased from apiaries that are located in northwestern Paraná state, Brazil. The samples were collected inside eucalyptus reserves that are surrounded by native forest with a predominance of *Baccharis dracunculifolia* (Asteraceae). Collection of samples occurred in 2005, 2008, and 2009 and they were named samples of Brazilian green propolis collected in 2005, samples of Brazilian green propolis collected in 2008, and samples of Brazilian green propolis collected in 2009, respectively.

PEs were prepared with a propolis/ethanol ratio of 30/70 (wt/wt) by turbo extraction and evaluated with regard to pH, density, and alcohol content. The resulting weight is presented as a dryness residue value. Six replicates were performed to estimate the inherent variability of each determination.[16] The resulting extracts were named PE-5, PE-8, and PE-9, corresponding to the samples of Brazilian green propolis collected in 2005, samples of Brazilian green propolis collected in 2008, and samples of Brazilian green propolis collected in 2009, respectively.

The total phenol content (TPC) of each PE was determined by the Folin-Ciocalteu method.[17,18] A calibration curve with solutions of gallic acid was used as a reference. The TPC is expressed as a percentage of total phenolic substances in the extract, corresponding to the mean of at least 6 replicates.

Antiherpetic Activity of PEs

Cells and Viruses

Vero cells (ATCC CCL-81, African green monkey kidney) were cultured in DMEM with 5%-10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37°C in 5% CO_2. The viral titers of KOS and AR-29 strains of HSV-1 were determined using the sulforhodamine B or plaque assay. Briefly, Vero cells (2.5×10^5 cells mL^{-1}) were seeded in 96- or 24-well plates and incubated for 24 h at 37°C in a CO_2 incubator. Cells were infected at a multiplicity of infection of 1 for the KOS and AR-29 strains.

Cytotoxicity and Anti-HSV-1 Activity of PEs

The cytotoxicity and antiherpetic activity of 3 samples of the PEs were determined using the sulforhodamine B colorimetric technique.[19] Briefly, confluent Vero cell monolayers were infected with HSV-1 KOS in $TCID_{50}$ for 1 h at 37°C. Afterward, the cells were exposed to different concentrations of the PEs for 72 h at 37°C in 5% CO_2. DMEM and serial dilutions of the PE (0.1, 1.0, 10, 100, 500, and 1000 μg mL^{-1}) were then added. After incubation, fixation was performed with 10% trichloroacetic acid for 1 h at 4°C, and the samples were stained with sulforhodamine B. Absorbance was determined in an enzyme-linked immunosorbent assay reader (Bio-Tek model Power Wave XS) at 530 nm. The concentration of each sample that reduced cell viability by 50% (CC_{50}) and the concentration that protected 50% (EC_{50}) of the cells were calculated based on untreated controls. EC_{50} and CC_{50} were determined by regression analysis of the concentration-response curves.

Mode of Action of PEs

Prophylactic Assay

Vero cell monolayers were treated with different concentrations of the PE for 1 and 2 h at 37°C in 5% CO_2. After incubation they were washed with phosphate-buffered saline (PBS) to remove the propolis-containing medium. The viruses (100 plaque forming unit [PFU] per well) were added and incubated for 1 h at 37°C in 5% CO_2. Unbound viruses were removed by washing with PBS and then the monolayers were overlaid with 2× DMEM that contained 0.5% carboxymethylcellulose (CMC medium) for 72 h at 37°C in 5% CO_2.[20]

Attachment and Penetration Assay

Prechilled Vero cell monolayers were exposed to viruses (100 PFU per well) in the presence or absence of the PE. After incubation for 1 h at 37°C, the samples and unabsorbed viruses were removed by washing with cold PBS, and the cells were overlaid with CMC medium for 72 h at 37°C in 5% CO_2.[21]

Attachment Assay

Vero cell monolayers were simultaneously exposed to different concentrations of the PE and viruses (100 PFU per well) for 1 h at 4°C. The samples and viruses were then removed by washing with cold PBS, and the cells were overlaid with CMC medium for 72 h at 37°C in 5% CO_2.[22]

Penetration Kinetics Assay

Viruses (100 PFU per well) were adsorbed for 1 h at 4°C on prechilled cells. After the removal of unbound viruses, the temperature was shifted to 37°C to allow penetration. The cells were then treated with different concentrations of prewarmed PE and incubated for 1 h at 37°C. Nonpenetrated viruses were inactivated with citrate buffer (pH 3.0), and the cells were washed with PBS and overlaid with CMC medium for 72 h at 37°C in 5% CO_2.[22,23]