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Antioxidant action of propolis on mouse lungs exposed to short-term cigarette smoke

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ABSTRACT

Propolis is a natural product with antioxidant properties. In this study, we tested the efficacy of propolis against acute lung inflammation (ALI) caused by cigarette smoke (CS). C57BL6 male mice were exposed to CS and treated with propolis (200 mg/kg orally, CS+P) or only with propolis (P). A Control group treated with propolis was sham-smoked (Control+P). We collected the lungs for histological and biochemical analyses. We observed an increase in alveolar macrophages and neutrophils in the CS group compared with the Control+P. These counts reduced in the CS+P group compared to the CS group. The treatment with propolis normalized all biochemical parameters in the CS+P group compared with the CS group, including nitrite, myeloperoxidase level, antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase), reduced glutathione/oxidized glutathione ratio and malondialdehyde. Additionally, TNF-α expression reduced in the CS+P group when compared with the CS group. These data imply a potential antioxidant and anti-inflammatory role for propolis with regard to ALI caused by CS in mice.

1. Introduction

Propolis (bee glue) is a resinous material produced by *Apis* that protects the hive against intruders. Some studies have reported that propolis contains phenolic-like compounds and esters, different types of flavonoids, fatty acids, terpenes, steroids, amino acids, polysaccharides, hydrocarbons and alcohols.[1–4] Propolis also has immunomodulatory,[5,6] anti-bacterial,[7] anti-viral,[8,9] anti-tumoral,[10] anti-ulcer,[1,11] and radioprotective properties.[12,13] Other studies have investigated the antioxidant properties of propolis and have reported that they were able to not only decrease lipid peroxidation and DNA damage[14,15] but also act as a free radical scavenger and reduce interferon-gamma (INF-γ) production.[5,16] Clinically, propolis has shown antioxidative actions without altering human blood parameters[17] or increasing respiratory parameters in asthmatic patients.[18] However, more studies are necessary to understand the mechanisms that are involved in the beneficial activities of propolis.

Cigarette smoke (CS) is composed of 4000 substances. CS is the primary risk factor for developing Chronic Obstructive Pulmonary Diseases (COPD).[19] Although acute lung inflammation (ALI) is caused by CS, but does not represent a good model of all the characteristics of COPD pathogenesis,[20] two processes act as hallmarks: accumulation of inflammatory cells[21] and lung oxidative stress.[22] CS contains oxidants (1014 radicals/cigarette puff)[23] that stimulate phagocytes to produce reactive oxygen species (ROS), such as hydrogen peroxide and radical hydroxyl, which generate lipid peroxidation in cells and promote NF-κB signaling[24] and TNF-α production.[25] Therefore, increasing our knowledge regarding the antioxidant properties of propolis is pivotal for improving treatment strategies for diseases related to tobacco dependence. The aim of the present study was to evaluate the anti-inflammatory and antioxidant actions of propolis against ALI caused by CS.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), bromophenol blue, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), eosin, glycerol, hematoxylin,

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hexadecyltrimethylammonium bromide (HTAB), 2-mercaptoethanol, naphthylenediamide dihydrochloride, nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione, phosphoric acid, reduced glutathione, sodium acetate, sodium dodecyl sulfate (SDS), sodium nitrite, sulfanilamide, 3,3′,5,5′-tetramethylbenzidine (TMB), triethanolamine, Tris–HCl, Tween 20, and 2-vinylpyridine were purchased from Sigma Chemical (St. Louis, MO, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Ethanol, formalin, hydrogen peroxide, potassium phosphate, and sodium chloride (NaCl) were purchased from Vetec (Duque de Caxias, Brazil). A commercial ethanolic extract of propolis (30% in ethanol) was purchased from Coapi (São Gonçalo, Brazil).

2.2. Chemical analysis of the ethanolic extract of propolis

We performed full-scan ESI-MS analyses using a MicrOTOF II mass spectrometer (Bruker Daltonics, Inc., Boston, MA, USA). We infused samples directly into the source at a flow rate of 0.12 mL/h. The source temperature was set at 180 °C, the drying gas (nitrogen) flow rate was 4.0 L/min and the nebulizer gas (nitrogen) pressure was 0.4 bar. In negative mode, the capillary voltage was 3.8 kV, the capillary exit voltage was −150 V, the skimmer 1 and 2 voltages were 50 V and 15 V, respectively, the hexapole 1 voltage was −23 V, the hexapole RF voltage was 300 Vpp, the lens 1 transfer was 88 μs and the lens 1 pre plus stage was 15 μs. Data were acquired in negative mode in the range of 50–2000 *m*/*z*. Mass calibration was achieved by infusing ammonium formate in an isopropanol–water mixture (1:1, v/v) as an external standard. All data were analyzed using Bruker Daltonics ESI Compass Data Analysis Version 4.0 SP 1 (Bruker Daltonics Inc., MA, USA). Mass error (the difference between measured and theoretical mass) and sigma (a parameter calculated by the software that accounts for the difference between theoretical and measured isotopic pattern; smaller values of sigma indicate better matching; data not shown) calculated for each datum.

2.3. Animals

Eight-week-old, male, C57BL/6 mice (18–22 g) were purchased from the Instituto de Veterinária—Universidade Federal Fluminense (Niterói, Brazil). The mice were fed Purina chow and allowed unrestricted access to water in a controlled environment maintained at 21 °C ± 2 °C, 54–56% relative humidity, and a 12-h light/dark cycle. The mice were allowed to acclimate for two weeks prior to the experimental procedures. The Ethics Committee from Instituto de Biologia Roberto Alcantara Gomes/Universidade do Estado do Rio de Janeiro approved the use of animals for this experiment (CEA). The CEA follows guidelines from the Intramural Animal Care and Use (ACU) program of the National Institutes of Health (NIH).

2.4. CS exposure and procedures

Thirty C57BL6 mice were divided into three groups (10 mice/group): a control group that was sham-smoked and treated with 200 mg/kg of propolis (100 μL),[26,27] a group that was exposed to CS and treated with vehicle (100 μL of ethanol) and a CS group treated with propolis (100 μL). All treatments were administered orally by gavage after last cigarette smoke exposure from each day. The ethanolic extract of propolis (200 mg/kg) was prepared daily and this dose was choosing after in vitro experiment of dose–response by using RAW 264.7 cells (data not shown). CS exposure was performed with 12 commercial full-flavored Marlboro cigarettes (10 mg tar, 0.9 mg nicotine, and 10 mg carbon monoxide) per day for 5 days with the use of a smoking chamber, as described previously.[28,29] Briefly, the animals were placed in the inhalation chamber (40 cm long, 30 cm wide and 25 cm high) inside an exhaust hood with the exhaust fan off. A cigarette was coupled to a plastic 60-mL syringe, and puffs of smoke were drawn into the syringe and then expelled into the inhalation chamber. One liter of smoke from each cigarette was aspirated with this syringe (20 puffs of 50 mL each), and each puff was immediately injected into the chamber. The animals were maintained in this smoke-filled environment (±3%) for 6 min. Then, the cover of the inhalation chamber was removed, and the exhaust fan of the hood was turned on to evacuate the smoke. The smoke was evacuated within 1 min. This exposure to CS was repeated four times (4 × 6 min) with an exhaust interval of 1 min after each exposure. We repeated this procedure three times per day (morning, noon and afternoon) which resulted in exposure to the smoke of twelve cigarettes over 72 min. Each cigarette produced 300 mg/m^3 of total particulate matter in the chamber (measured by weighing the material collected on Pallflex filters). Carboxyhemoglobin (COHb) levels were measured to confirm that the treatment was not toxic, as described previously.[30]

2.5. Broncoalveolar lavage and lung homogenates

Twenty-four hours after CS exposure and propolis treatment, the mice were sacrificed by cervical dislocation. A broncoalveolar lavage (BAL) was performed in the left lung of each animal. Briefly, the right lung was clamped, and a cannula was inserted into the trachea. The airspaces were washed with buffered saline solution (500 μL) three times and the flow-through (final volume 1.2–1.5 mL) was maintained on ice. Next, the BAL fluid was centrifuged, and the supernatant was collected and stored on ice for subsequent analyses of nitrite and MPO content. Then, the left lungs of each group were removed and immediately homogenized (Homogenizer NovaTécnica mod NT 136, Piracicaba, Brazil) in 1.0 mL potassium phosphate buffer (pH 7.5). The homogenates were centrifuged at 7000*g* (Centrifuge FANEM mod 243 M, São Paulo, Brazil) for 10 min, and the supernatants were stored at −20 °C for analyses of SOD, CAT, GPx, GSH/GSSG ratio, malondialdehyde and TNF-α expression. The total protein in the samples (tissues and BAL) was determined by the Bradford method.[31]

2.6. Tissue processing and morphometry

The right ventricles of all mice were perfused with saline to remove the blood. Next, the right lungs of all animals were inflated with 4% phosphate buffered formalin (pH 7.2) at 25 cm H_2O pressure for 2 min and then ligated, removed and weighed. The inflated lungs were fixed for 48 h before embedding in paraffin. Sagittal, 4-μm serial sections of the right lungs were stained with hematoxylin and eosin (H&E) for histological analyses. Alveolar macrophage and neutrophil numbers were estimated by counting ten different random fields (5 random fields from 2 different sections) per lung. Morphometry was performed by using a microscope (20× objective lens) and total area analyzed was ∼2 mm^2/lung. Two investigators performed morphometry by counting coded sections.

2.7. Nitrite content

The nitrite contents in the BAL fluid were determined using a method based on the Griess reaction.[32] A quantity of 100 μL of sample was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylenediamide dihydrochloride in water) and was incubated at room temperature for 10 min. The absorbance was measured with a plate reader at 550 nm (Bio-Rad Microplate Reader model 680, Hercules, CA, USA). Nitrite concentrations in the samples were determined from