Effect of propolis on proliferation and apoptosis of periodontal ligament fibroblasts

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The most critical factors affecting the prognosis of an avulsed tooth are extraoral dry time and storage media used before replantation. Studies have analyzed different storage media to determine the ideal solution to preserve periodontal ligament (PDL) cell viability. Propolis has anti-inflammatory and antimicrobial properties, and has been previously suggested as a storage medium. The purpose of this study was to assess not only cell viability but also physiological health of PDL cells after exposure to propolis. PDL cells were exposed to different concentrations of propolis or Hanks balanced salt solution, and the apoptotic levels were determined using apoptosis assay and flow cytometry. Additional cell viability and proliferation were analyzed by XXT assay in dry and wet conditions. Propolis not only decreased apoptosis but also increased the metabolic activity and proliferation of PDL cells. This study suggests that propolis is a suitable storage medium for avulsed teeth. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:843-848)**

Tooth avulsion is characterized by complete displacement of the tooth from its alveolar socket. Avulsion accounts for up to 16% of all dental traumatic injuries in the permanent dentition.[1] Studies have revealed that the prevalence of dental traumatic injuries is increasing, ranging from 16% to 40% among 6-year-old children and from 4% to 33% among 12- to 14-year-old children.[2] A significant proportion of dental trauma relates to sports, unsafe playgrounds or schools, road accidents, or violence. The prevalence of trauma is higher in boys (19.3%) than in girls (9.7%), and is higher in the maxilla (13.6%) than in the mandible (1.5%). Most of the affected subjects (77.3%) have had only one injured tooth, and most of the traumatized teeth were maxillary central incisors (83.7%).[2,3]

Immediate replantation of an avulsed tooth is the best treatment option at the site of the accident; however, this is not always possible.[4] The 2 major causes of failure after replantation are inflammatory root resorption and replacement resorption.[5] As a consequence of the total displacement of the tooth from its alveolus, the neurovascular supply is severely compromised, which usually results in loss of vascularization and pulp necrosis. When the tooth is separated from the socket, tearing of the periodontal ligament (PDL) leaves viable cells along the root surface. In cases of large areas of PDL damage, competitive wound healing begins between cells programmed to form bone and PDL-derived cells programmed to form PDL fibers and cementum. This competitive wound healing might lead to replacement resorption or ankylosis after tooth replantation. If excessive drying occurs before replantation, however, the damaged PDL cells will elicit a severe inflammatory response over the root surface, resulting in inflammatory root resorption.[5]

Avulsion illicits immune responses in all the tissues involved, including the PDL cells, and apoptosis can be one of these responses. This programmed cell death is characterized by a variety of morphologic features, such as loss of plasma membrane lipid asymmetry, nuclear condensation, cell shrinkage, and DNA fragmentation. Under physiological conditions, apoptosis is critical for the turnover of cells in tissues, and during normal development and senescence; however, a detrimental injury to the cell, such as toxic substances or physical damage, can quickly activate the apoptotic response.[6]

Studies have shown that the most critical factors affecting the prognosis of an avulsed tooth after replantation are the extraoral dry time, the amount of physical damage to the root surface, and the storage medium in which the avulsed tooth is stored before treatment.[7,8] Extraoral dry time and damage to the root surface are aspects that can be controlled only to a certain extent; therefore, to minimize failure after replantation, the

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Received for publication Jan 19, 2011; returned for revision Aug 11, 2011; accepted for publication Aug 11, 2011.
1079-2104/$ - see front matter

doi:10.1016/j.tripleo.2011.08.004

avulsed tooth should be stored in a solution that preserves the viability of PDL cells.

Several storage media have been suggested for an avulsed tooth, including Hanks Balanced Salt Solution (HBSS), milk, or saline.[9-11] Earlier studies even suggested placing the tooth under the patient's tongue in cases in which immediate replantation is not possible.[12]

Propolis is a natural, brownish resinous substance collected by bees, mainly from poplar and conifer buds, and is used to seal their hives. Propolis has been used in medicine because of its anti-inflammatory, antiseptic, antimicrobial, and healing properties.[13-15] Interestingly, Martin and Pileggi[16] demonstrated that propolis was able to maintain PDL cell viability better than HBSS, milk, or saline, and could be a promising new storage medium following tooth avulsion. This study evaluated cell viability by trypan blue counting, which assesses only vital cells but not the physiological health or metabolic capacities of PDL cells. The health status of viable PDL cells is likely critical to prevention of resorptive sequelae after replantation.[16]

To date, the physiological health in addition to the apoptotic status of periodontal ligament cells following storage in different media has not been investigated. Therefore, the purpose of the present study was to analyze proliferation and apoptosis of PDL cells following storage in propolis or HBSS.

MATERIAL AND METHODS

Cell culture

Human PDL fibroblast cells (Cambrex Corporation, East Rutherford, NJ) were cultured in fibroblast growth medium (Cambrex) supplemented with 1 mg/mL human recombinant fibroblast growth factor, 5 mg/mL insulin, 50 mg/mL gentamicin, 50 mg/mL amphotericin-B (provided by Cambrex bullet kit), and 10% fetal bovine serum (FBS). Cells of the third to sixth passage were exposed to HBSS, propolis, or vehicle control. Processed propolis was obtained from southeastern Brazil and ground into fine particles with a mortar and pestle. Propolis was prepared at final concentrations of 25%, 50%, or 100% in 0.4% ethanol/HBSS solution.

Apoptosis assay

The Vybrant Apoptosis Assay Kit #10 (Invitrogen Corporation, Carlsbad, CA) was used to assess viable, nonviable, and apoptotic cells. PDL cells were cultured in flasks, medium was aspirated, and cells were detached using the trypsin/EDTA solution (Cambrex). Cells were exposed to the experimental medium for 1.5 or 3.0 hours to simulate the amount of time an avulsed tooth might remain in storage medium. Treatment groups consisted of the following: (1) 50% propolis solution; (2) HBSS; (3) cell culture medium/10% FBS. HEPES and 2-mmol/L hydrogen peroxide were used as negative and positive controls, respectively. Assay was performed according to the manufacturer's instructions. Briefly, annexin-binding buffer, 1 mmol/L stock solution of C_{12}-resazurin, and 1 μmol/L working solution of SYTOX Green stain were prepared. Cells were washed in annexin-binding buffer and pelleted by centrifugation. Supernatants were discarded and cells were resuspended in annexin-binding buffer. Five microliters of allophycocyanin annexin V, 1 μL of C_{12}-resazurin working solution (50 μmol/L), and 1 μL of SYTOX Green stain working solution (1 μmol/L) were added to each 100 μL of cell suspension. Cells were incubated at 37°C in an atmosphere of 5% CO_2 for 15 minutes. After incubation, 400 μL of annexin-binding buffer was added and stained cells were analyzed by flow cytometry. Fluorescence emission was measured at 530 nm and 575 nm using 488-nm excitation; and at 660 nm using 633-nm excitation.

Cell proliferation assay

PDL cells were counted using a hemocytometer and plated in 96-well tissue culture plates in 100 μL of DMEM/10% FBS containing 10^4 cells/mL in each well. Plates were incubated at 37°C in 5% CO_2 and 95% humidity for 24 hours to allow cell attachment. Medium was removed from half of the wells after 24 hours, and plates were placed at the laboratory bench for 30 minutes to simulate extraoral dry time following avulsion. Medium was aspirated from the remaining wells and 100 μL of experimental medium was added to each well (triplicate). Treatment groups consisted of the following: (1) HBSS; (2) propolis 100%; (3) propolis 50%; (4) propolis 25%; (5) 0.4% ethanol/HBSS solution (vehicle, negative control); and (6) cell culture medium (DMEM/10% FBS, positive control). Cells were exposed to the experimental solutions for 1.5 or 3.0 hours. Solutions were aspirated and XTT assay (Roche, Mannheim, Germany) was performed according to the manufacturer's instructions. Briefly, XTT reagents were added to each well and incubated at 37°C for 4 hours to generate colorimetric formazan products. Absorbance of formazan products was measured by optical density at a wavelength of 450 nm with reference wavelength of 630 nm after 2, 3, and 4 hours. The assay was performed 3 times on 3 different days.

Statistical analysis

Results of cell proliferation assays were analyzed by 2×4 general linear model and 2-tailed Dunnett's post hoc tests. Results of the apoptosis assays were analyzed using univariate t test ($P \leq .05$).