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 incites 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
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.\textsuperscript{9-11} Earlier studies even suggested placing the tooth under the patient’s tongue in cases in which immediate replantation is not possible.\textsuperscript{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.\textsuperscript{13-15} Interestingly, Martin and Pileggi\textsuperscript{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.\textsuperscript{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 the experimental solutions for 1.5 or 3.0 hours. Solutions were aspirated and XTT assay was performed 3 times on 3 different days. 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 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\textsubscript{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\textsubscript{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\textsubscript{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 ≤ .05).
RESULTS

Apoptosis assays showed that periodontal ligament fibroblasts exposed to propolis 50% presented fewer apoptotic cells than HBSS (Fig. 1, A and B). There was no significant difference in the apoptotic levels of PDL cells when exposed to propolis 50% or 100% (data not shown).

Periodontal ligament fibroblasts showed statistically significant increase in cell proliferation when exposed to propolis 50% and 100% as compared with PDL cells exposed to HBSS or control analyzed by XTT assays. As expected, all wet treatments demonstrated increased cell proliferation activity as compared with dry treatments. Notably, storage in propolis 50% and 100% demonstrated the most significant difference between wet and dry treatments. There was no significant difference between HBSS, propolis 25%, or control (Fig. 2, A and B).

DISCUSSION

Many authors have investigated factors contributing to the success of replanted teeth; more specifically, the factors involved in maintaining periodontal ligament cell viability following an avulsion injury. Several storage media have been suggested to preserve the vitality of PDL cells. Saliva and tap water have been shown to cause deleterious effects on PDL cells, whereas milk has been demonstrated to be a suitable storage medium. Milk has a physiological osmolality, as opposed to water and saliva, and contains markedly fewer bacteria. In accordance with this study, milk was suggested to be a significantly better preservative of PDL fibroblast viability than saliva, water, or air-drying, but not as good as HBSS. In fact, there were 50% more viable PDL cells after storage in HBSS than in milk, and 10 to 50 times more viable cells following storage in HBSS than in saliva or water. Viaspan has been suggested as the most effective storage medium compared with milk and HBSS, maintaining 37% of the cells viable after 168 hours. This study has shown that the number of viable cells stored in milk had dropped from 76.7% at 2 hours to 43.4% at 12 hours, whereas cells stored in HBSS and Viaspan maintained 72.9% and 70.5% vitality, respectively.

Preservation of the physiological and metabolic health of PDL cells depends on several properties of the storage medium, namely compatible pH, osmolality, and availability of cell metabolites. The storage environment of the avulsed tooth should mimic the oral environment as closely as possible. Therefore, replacement of depleted metabolites, physiological pH and osmolality, and temperature are properties that should be considered when evaluating storage media. In the current study, propolis solutions were prepared with HBSS, which has a physiological pH and osmolality, contains glucose as an energy source, and cell metabolites, including sodium, calcium, potassium, and phosphate. The purpose of using propolis with HBSS was to combine the properties of a well-established storage medium with the anti-inflammatory, antiseptic, and antimicrobial properties of a natural product, aiming to increase not only the viability but also the physiological health of PDL cells.

Propolis has been used in folk and holistic medicine for many years to treat a variety of diseases; however, unlike many other holistic compounds, there has been extensive research on propolis, particularly in the medical community. Propolis has been shown to have minimal toxicity on both PDL and pulp fibroblasts; moreover, propolis has proved to be significantly less cytotoxic to PDL cells than HBSS and milk.
The present study extended the work of Martin and Pileggi, which demonstrated that propolis 50% was significantly more effective in maintaining PDL cell viability than milk, saline, or HBSS. In the previous study, periodontal ligament cells were collected from freshly extracted teeth and cell viability was determined by trypan blue dye exclusion. Although this assay can determine cell viability, the physiological health or proliferative capacity cannot be assessed. Another limitation is that the minimum number of cells needed to
perform proliferation and apoptotic assays exceeds the number of cells provided by a single tooth. Therefore, considering these limitations and the time-sensitive nature of this study, primary cultures of periodontal ligament fibroblasts were used here. This not only allowed the present study to be performed strictly according to time constraint protocols, but also provided better standardization, avoiding the variabilities of tooth extraction techniques and patients.

Many studies have examined the critical dry time before irreparable damage to the PDL cells has occurred. Teeth replanted within 30 minutes have a better success rate than those that were extraoral for longer periods. In addition, other investigators have shown that after 2 hours of dry time, no vital PDL cells remain viable. In the present study, a 30-minute dry time was chosen, because this seems to be a critical time at which damage has been done to many PDL cells; yet some cells remain for assessment. Also, 30 minutes represents a typical clinical scenario during which the avulsed tooth may remain dry before being placed into a storage medium. Another issue is the time period in which the avulsed tooth is maintained in storage medium. Studies have shown that PDL cells can remain viable following 1 to 3 hours in storage medium. Therefore, we selected the 1.5- to 3.0-hour time frame for our studies to establish a baseline to evaluate the effect of propolis in rescuing damaged PDL cells. Future studies would determine whether propolis increases the viability and physiological health of PDL cells over an extended period.

Various methods have been used to assess proliferation and viability of PDL cells following storage in medium, such as neutral red or the trypan blue dye exclusion assay, fluorescein diacetate staining, Cell-Titer 96 AQ assay (Promega Corp, Madison, WI, USA), and the clonogenic and mitogenic assay (thymidine incorporation). Here, we used the Vybrant apoptosis assay to determine cell viability. This assay detects one of the earliest stages of apoptosis, which is the translocation of phosphatidylserine (PS), a membrane phospholipid, from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. Therefore, this method provides a more suitable assessment of cell viability than dye exclusion techniques. It was critical for us to demonstrate not only the apoptotic levels, but also the physiological health of the PDL cells. Therefore, we used the XTT assay to assess cellular proliferation and viability. The measurement of mitochondrial dehydrogenase activity by XTT provides additional information about the physiological condition of the cell as compared with dye exclusion techniques.

To date, the physiological health of periodontal ligament fibroblasts following storage in different media has not been reported in the dental trauma literature. Thus, the present study explored not only apoptosis of PDL cells, but also their metabolic activity following storage in propolis or HBSS.

**CONCLUSIONS**

According to the results presented here, we conclude that propolis decreased the apoptotic levels of periodontal ligament fibroblasts as compared with HBSS. Furthermore, propolis increased mitochondrial enzymatic activity of PDL cells when compared with HBSS alone. Collectively, our results suggest that propolis solution can be a more beneficial storage medium for an avulsed tooth, increasing not only the viability but also the physiological health of PDL cells.

**REFERENCES**


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