Effect of Propolis on Survival of Periodontal Ligament Cells: New Storage Media for Avulsed Teeth

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Abstract

Propolis is a multifunctional material used by bees in the construction and maintenance of their hives. Propolis possesses several biologic activities such as anti-inflammatory, antibacterial, antioxidant, antifungal, antiviral, and tissue regenerative, among others. The purpose of this study was to determine the ability of propolis to serve as a temporary storage medium for the maintenance of periodontal ligament (PDL) cell viability of avulsed teeth. PDL cells were obtained from healthy third molars and cultured in Dulbecco's Modified Eagles Medium (DMEM). Cultures were subjected to 10% propolis solution, 20% propolis solution, long-shelf life light milk with lower fat content (milk), Hank's Balanced Salt Solution, tap water as the negative control, and DMEM as the positive control. Tissue culture plates were incubated with experimental media at 37°C for 1, 3, 6, 12, or 24 hours. PDL cell viability was assessed by trypan blue exclusion. Statistical analysis of the data was accomplished by using one-way analysis of variance complemented by the Tukey test. The level of significance was 5% ($p < 0.05$). The results showed that 10% propolis was a more effective storage medium than other groups. In conclusion, propolis can be recommended as a suitable transport medium for avulsed teeth. *(J Endod 2007;33: 570–573)*

Key Words

Cell culture, propolis, storage media

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The reported incidence of complete avulsion ranges from 1% to 16% of all traumatic injuries to the permanent dentition (1). When avulsion occurs, the avulsed tooth should be immediately replanted at the site of the accident to prevent further damage to the PDL cells from desiccation. However, immediate repositioning of teeth is not always possible under certain conditions. In such a case, storage medium is used to preserve PDL cell viability. The choice of a suitable storage medium for maintenance of maximum PDL cell survival until replantation is very important.

In 1994, the American Association of Endodontists recommended Hank's balanced salt solution (HBSS) as the storage medium of choice for avulsed teeth (2). Milk has been studied extensively (3–6) and has gained wide acceptance as a medium capable of maintaining PDL cell viability. Harkacz et al. (5) showed that the fat content of milk had an effect on PDL cell viability. They suggested that milk with a lower fat content might be more appropriate at maintaining PDL cell viability than milk with a higher fat content.

Propolis, a substance made by the honeybee, is a potent antimicrobial, antioxidant, and anti-inflammatory agent. Recently, it has attracted much attention as a useful substance applied in medicine and cosmetics because of its antibacterial and antifungal activities (7). In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% various other substances including organic debris depending on the place and time of collection (8, 9). The constituents of propolis vary widely because of climate, season, location, and year, and its chemical formula is not stable (10, 11).

In this study, we compared HBSS and milk with natural product propolis on enhancement of PDL cell viability.

Materials and Methods

Preparation of Dimethyl Sulfoxide Extract of Propolis

Propolis was produced by honeybees (*Apis mellifera L*) in Trabzon, Turkey. It was provided by Trabzon Agricultural Development Cooperative. In pre-experimental studies, alcoholic extracts showed cytotoxicity for cancer as well as control cell lines. Therefore, we used an alternative extraction procedure by using dimethyl sulfoxide as solvent. Propolis sample was grinded (Retsch, ZM 200, Retsch Technology, Haan, Germany) and bottled in a 10-g portion. It was dissolved in 20 mL dimethyl sulfoxide (100% w/v) by a magnetic mixer for 24 hours at 37°C. Then rough particles were removed from propolis extract by filter. Dulbecco's Modified Eagles Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was added until propolis extract concentration at 10% and 20% was achieved.

Primary Culture of Human Periodontal Ligament Cells

The PDL cells were obtained from clinically healthy third-molar teeth extracted for orthodontic purposes. The teeth were extracted as atraumatically as possible and washed in sterile saline solution to eliminate the residual blood. PDL tissues were scraped with a #15 scalpel using aseptic techniques and then transferred to culture medium.

The PDL samples were cut into 1- to 2-mm^3 pieces and then washed twice with Hank's salt solution. Thereafter, the cut biopsies were placed into tissue culture flasks (25 cm^2). The explants were incubated with culture medium consisting of DMEM, 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glucose (4.5 g/L),

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$NaHCO_3$ (3.7 g/L), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin (2.5 mg/mL; all from Biochrom KG, Berlin, Germany) supplemented with 10% heat inactivated fetal calf serum (Pan Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidified atmosphere of 10% CO_2 in air. Culture medium was renewed twice per week until cells reached confluency. For subcultivation, cells were detached from the culture flasks with 0.25% Trypsin/EDTA Solution (Sigma) for 3 to 5 minutes. Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages. Cell morphology was visualized with phase contrast microscopy (TNM, Nikon, Tokyo, Japan).

Exposure of PDL Cultures to Different Solutions

On the day of treatment, the culture medium was drained from each well, and the cells exposed to 2 mL of the different experimental solution. The storage solutions used in the experiments were as follows: (1) containing DMEM 10% propolis, (2) containing DMEM 20% propolis, (3) milk (UHT skimmed milk with 0.12% fat content; Pınar Dairy Products Inc., Istanbul, Turkey), (4) HBSS, (5) cultures grown on fresh DMEM, and (6) tap water.

Assessing the Viability of Cells by Trypan Blue Exclusion

After exposure, the medium was removed from the wells after 1, 3, 6, 12, and 24 hours washed with 2 mL sterile phosphate-buffered saline, 100 μL 0.25% trypsin was added, and the plates were incubated at 37°C for 5 to 10 minutes. Full-growth medium (50 μL) and 0.4% trypan blue (50 μL) were added to each well, and the plates were returned to the incubator for another 5 minutes. After this time, a 20-μL aliquot was removed and placed under a cover slip on a hemocytometer, and both the viable and the nonviable cells were counted under the microscope. The number of viable cells harvested from each petri dish was obtained by the following mathematical equation: $UC \times D \times 10^4/\#SQ$, where UC = unstained cell count (viable cells), D = the dilution of the cell suspension, and #SQ = number of squares of the hemocytometer counted. The viability percentage of the cell population of each Petri dish was obtained by applying the following mathematical equation: $UC/TC \times 100$, where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells). Each experiment was repeated four times.

Statistical Analysis

Statistical analysis of the data was accomplished by using one-way analysis of variance complemented by the Tukey test. The level of significance was 5% ($p < 0.05$).

Results

The mean percentage values of cell viability are shown in Table 1. The efficacy of 10% propolis at 3, 6, 12, and 24 hours was found significantly better than HBSS and milk ($p < 0.05$). Whereas, at 1 hour, there was no significant difference between them. At 1 hour, 20% propolis was found significantly worse then HBSS; on the other hand, at 3 and 6 hours. 20% propolis was found significantly better than HBSS and milk. Whereas, at 12 and 24 hours, 20% propolis was found significantly only better than HBSS. When propolis solutions were compared, there was no difference at 1, 3, and 6 hours. Whereas, at 12 and 24 hours, 10% propolis was found significantly better than 20% propolis. Figure 1 shows time-dependent results according to experimental solutions on cell viability.

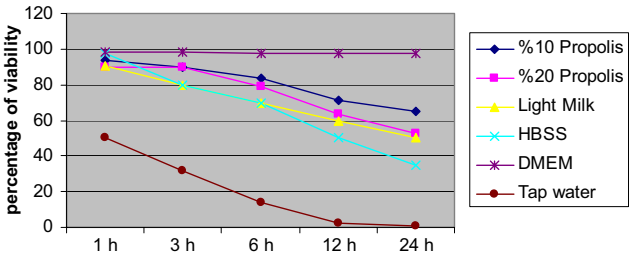


Figure 1. PDL cell viability.

Discussion

Avulsion injuries are the worst of the dentoalveolar injuries. Usually, avulsion involves a single tooth, and the most frequently avulsed tooth is the maxillary central incisor (12). The treatment of such injuries must be geared toward early reestablishment of PDL cellular physiology.

Successful replantation of avulsed teeth is dependent on the prevention or limitation of inflammatory and replacement root resorption (ankylosis) (13, 14). Clinical studies have shown that inflammatory resorption has an early onset, progresses rapidly, and is associated with pulpal infection, contamination of the root surface, and an extraoral dry time of 15 minutes or more (13, 15–18). Inhibition of inflammatory resorption involves removing the necrotic tissue and filling the root canal with calcium hydroxide (12). Replacement resorption may have a delayed onset and has been correlated with extensive PDL damage, a lengthy extraoral storage time and prolonged or rigid splinting (17–20).

Human PDL fibroblasts behave differently in cell culture than gingival fibroblasts with respect to growth rates and to protein and collagen production (21). Although lip, gingival, and PDL fibroblasts have similar morphology; they do not behave the same in culture (22). Protein and collagen production are significantly greater in PDL cells and growth rates differ (22). Fibroblast cells obtained from the human PDL may more accurately reflect the ability of PDL cells on an avulsed tooth to remain viable in a transport medium than fibroblasts obtained from other sources. In view of this information, we used a human PDL fibroblast cell-culture model for evaluation of the efficacy of propolis solutions in maintaining the viability of PDL cells on avulsed teeth.

We chose the trypan blue exclusion staining technique because it is quick, easily performed, and distinctively differentiates nonviable cells from viable cells. However, the health of the viable cells and their ability to proliferate cannot be determined from this technique. We

TABLE 1. Mean percentage of cell viability

Medium	1 h	3 h	6 h	12 h	24 h
10% propolis	93.75 (3.5)	90.00 (3.55)	83.50 (1.29)	71.25 (4.78)	65.00 (4.76)
20% propolis	90.00 (4.39)	90.00 (4.39)	79.25 (3.86)	63.25 (3.40)	53.00 (2.16)
Milk	90.50 (4.20)	80.00 (4.76)	70.00 (4.39)	60.00 (3.55)	50.00 (3.55)
HBSS	97.50 (1.73)	80.00 (4.08)	70.00 (3.55)	50.00 (3.55)	35.00 (4.76)
DMEM	98.25 (0.95)	98.25 (0.95)	97.25 (1.70)	97.5 (1.29)	97.50 (1.29)
Tap water	50.25 (2.5)	31.50 (5.44)	13.75 (5.67)	2.0 (1.2)	0.75 (0.95)

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simply aimed to investigate the effects of experimental solutions on viability of PDL cells.

Up to now, several studies have examined the usefulness of several storage media. Results indicated that tap water, saliva, and saline were all ineffective for the maintenance of PDL cell viability. These media are no longer recommended because of their hypotonic properties of high incidence of bacterial contamination leading to the rapid death of the PDL cells (23).

Hank's balanced salt solution (HBSS) is a standard saline solution that is widely used in biomedical research to support the growth of many cell types (24). It is nontoxic, pH balanced, and contains many essential nutrients (25, 26). A tooth-preserving system using HBSS as a storage medium was developed and has become commercially available as Save-A-Tooth (Save-A-Tooth Inc., Pottstown, PA) (24), although it is not yet widely available in pharmacies or drug stores.

Milk has been shown to maintain human PDL fibroblast viability (3–5,27) and is accepted by the American Association of Endodontists as a suitable transport medium for avulsed teeth (2). The improved viability of cells stored in milk may be caused by the physiologic osmolality of milk (27–29). There is evidence that milk with a lower fat content may be more appropriate at maintaining cell viability than milk with a higher fat content (5). Our results agreed with those of the previous studies that suggested milk could be used as a storage medium for PDL cells. In addition, we used long-shelf life milk with lower content that can be readily available in locations where avulsions usually occur.

Propolis, a substance made by the honeybee, is a potent antimicrobial and anti-inflammatory agent. Solution form of propolis has never been tested for PDL cell viability up to now. In this study, two different concentrations of propolis were compared with HBSS, long-shelf life milk with lower fat content, and tap water. Both propolis groups showed more viable PDL cells than HBSS, milk, or tap water. At 12 and 24 hours, propolis at a lower concentration showed better results than at a higher concentration.

Oxygen radicals and oxygen tension have been reported to modulate osteoblast and osteoclast activities (30, 31). Buttke and Trope (32) stated that low levels of H_2O_2 in media used for storing avulsed teeth might adversely affect cells of the attachment apparatus. Oxidative damage may promote root-surface resorption via toxic effects on mechanically damaged cells of the PDL or cementum or by enhancing the resorptive activity of clastic cells (32). They also suggested that storing avulsed teeth in a medium containing one or more antioxidants might increase replantation success. Up to now, several studies (33–36) revealed that propolis has a strong antioxidant capacity. A major component of propolis is flavanoids. Flavanoids are powerful antioxidants, and they have been shown to be capable of scavenging-free radicals and thereby protecting against lipid peroxidation in the cell membrane.

Several studies showed favorable effects of antibiotics either topical or systemic on avoiding root resorption after replantation. The use of systemic penicillin after an avulsion injury has been recommended to decrease the occurrence of resorption complications. Tetracycline antibiotics have been reported to possess antiresorptive properties in addition to their antimicrobial actions (37). Previous studies have indicated that inflammatory resorption and ankylosis, which are frequent sequelae after delayed tooth replantation, can be greatly reduced by treating the root surface with 1% solutions of stannous fluoride and tetracycline (38). Because propolis has antimicrobial properties, this can make propolis a favorable storage media.

Based on the favorable results obtained in this study, propolis can be recommended as a suitable transport medium for avulsed teeth. We agree with Martin and Pileggi (39) that propolis not only keeps PDL cells alive but also has antimicrobial, anti-inflammatory, and antioxidant abilities. These benefits of propolis make it very favorable. Future research on animal models can show its effects on replanted teeth clearly. Because storage medium of propolis is not available, milk and HBSS are still the first choice.

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