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A quantitative analysis of Propolis: a promising new storage media following avulsion

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Abstract – Both length of extra-alveolar time and type of storage media are significant factors that can affect the long-term prognosis of replanted teeth. Numerous studies have examined various media in an attempt to determine the ideal material for storage of the avulsed tooth. The purpose of this study was to use a Collagenase–Dispase assay to investigate the potential of a new storage media, Propolis, in maintaining viable periodontal ligament (PDL) cells on simulated avulsed teeth. Seventy freshly extracted human teeth were divided into five experimental groups and two control groups. The positive and negative controls corresponded to 0-min and an 8-h dry time, respectively. The experimental teeth were stored dry for 30 min and then immersed in one of the five media (Hank's balanced salt solution (HBSS), milk, saline, Propolis 50%, and Propolis 100% for 45 min). The teeth were then treated with dispase grade II and collagenase for 30 min. The number of viable PDL cells were counted with a hemocytometer and analyzed. Statistical analysis demonstrated that both Propolis groups kept significantly more PDL cells viable compared to either milk, saline, or HBSS. Within the parameters of this study, it appears that Propolis may be a better alternative to HBSS, milk, or saline in terms of maintaining PDL cell viability after avulsion and storage.

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Traumatic injuries are a frequent occurrence today. Clinical surveys indicate that traumatic dental injuries in children and adolescents are a common problem, and several studies have shown that the prevalence of these injuries is increasing (1). Andreasen & Andreasen (2) predicted that the incidence of these injuries may eventually surpass the incidence of dental caries. The practitioner needs to be ready to manage these injuries and the various complicating factors indicated.

An avulsion injury results in complete displacement from the socket. In addition, the neurovascular supply is severely compromised, which usually results in loss of vascularization to the pulp. After an avulsion trauma, the success postreplantation is dependent upon the immediate treatment following injury (3, 4). Therefore, the clinician must be aware of possible complications after treatment and understand how to minimize their incidence. It has been well established that two major sequelae leading to failure postreplantation is that of inflammatory root resorption and replacement resorption. Furthermore, the literature has shown that two of the most critical factors affecting the prognosis of an avulsed tooth after replantation are extraoral dry time and the storage media in which the tooth is placed before treatment is rendered. According to Andreasen et al. (5, 6), the factors that play a role in the healing of the periodontal ligament (PDL) after avulsion injuries are primarily the amount of physical damage to the root surface and the type of medium in which the avulsed tooth is stored. The goal of treating the patient with an avulsed tooth is to replant the tooth immediately, but as this is not always possible, storage in solutions that preserve the PDL cell vitality should be used to help prevent the sequelae

of inflammatory root resorption and replacement resorption.

Several methods have been suggested to preserve the vitality of the PDL cells. Axhausen (7) suggested placing the tooth under the patient's tongue in cases in which immediate replantation was not possible. However, Dumsha (3), and Patil et al. (8) suggested storing the avulsed tooth in milk, Hank's Balanced Salt Solution (HBSS), or saline. Currently, no studies have suggested using Propolis as a storage media. Propolis is an inherently antibacterial and anti-inflammatory resinous bee-hive product (9). These qualities may be beneficial to the avulsed tooth.

Several techniques have been used to determine the viability of the PDL cells following avulsion. However, most of the experimental data that are available have been obtained using techniques in which the cells are cultured and/or trypsinized for longer periods of time. As the extracellular matrix has a high content of collagen and other proteins, it seems reasonable that the use of enzymatic desegregation would provide a greater number of cells within a shorter time frame.

Pileggi et al. used a collagenase dispase assay to compare PDL viability of simulated avulsed teeth after storage in HBSS, milk, saline, or water. Both collagenase and dispase enzymes disrupt the extracellular matrix and cause the release of cells without excessive disruption and destruction of their own membrane. Therefore, these authors suggested that the use of these two enzymes may provide additional data regarding the viability of PDL cells after avulsion injury. Furthermore, this method may be more representative of the actual clinical situation because the cells are not subjected to long processing times to determine their viability status (10).

The purpose of this study was to investigate a potentially new storage media, Propolis, in maintaining viable PDL cells after a simulated avulsion injury.

Material and methods

Seventy freshly extracted single-rooted human teeth with closed apices were obtained for this study. Teeth extracted from patients with moderate to severe periodontal disease or with extensive caries were excluded. The average age of the patient was 45 years. Extractions were performed as atraumatically as possible by an oral surgery resident. Following extractions, the teeth were held with forceps by the coronal region, and the coronal 3 mm of PDL was scraped with a curette to remove cells that may have been damaged.

The teeth were then randomly divided into one of the five experimental storage solution groups, with 12 samples per group, or a positive or negative control group, consisting of five samples each. The teeth in the experimental groups were dried for 30 min (during which time, the coronal PDL cells were curetted), followed by a 45-min immersion in one of the five following experimental media: saline, milk, HBSS, Propolis 50%, and Propolis 100%. Neither the positive control teeth after extraction were dried, nor were they stored in any solution, but rather they were immediately treated with dispase and collagenase. The negative control teeth were bench-dried for 8 h, with no follow-up storage solution time, and then placed in the dispase and collagenase.

Processed Propolis from South-eastern Brazil was obtained. Solid Propolis was ground into fine particles with a mortar and pestle. Propolis was then made into two different concentrations within a 0.4% ethanol solution. Propolis 50% consisted of 50 mg ground Propolis per 250 ml of the 0.4% ethanol solution, whereas Propolis 100% was made with 100 mg ground Propolis per 250 ml of ethanol solution. Before submersion of teeth in Propolis, the solutions were shaken for 15 min.

Each experimental tooth, after drying and soaking, was incubated for 30 min in 15 ml Falcon tubes with a 2.5 ml solution of 0.2 mg ml^{-1} of collagenase CLS II (Cooper Biomedical, PA, USA) and a 2.4 mg ml^{-1} solution of dispase grade II (Gibco, Taastrup, Denmark) in phosphate buffer saline (PBS). After incubation, 50 µl of fetal bovine serum (FBS) was added to each tube. All tubes were then centrifuged for 4 min at 1000 r.p.m. The supernatant was then removed with sterile micropipettes, and the cells were labeled with 0.4% Trypan Blue (Gibco BRL, Taastrup, Denmark) for determination of viability, according to Polverini & Leibovich (11). The number of viable protective least significant difference (PDL) cells were counted under a light microscope with a hemocytometer at 20× magnification. The results were statistically analyzed with an ANOVA and a post hoc Fischer's PLSD test.

Results

The ANOVA demonstrated a significant difference among the groups (Table 1). Table 2 presents the mean

Table 1. ANOVA table for 10E4 cells ml^{-1}

	df	Sum of squares	Mean square	*F*-value	*P*-value	Lambda	Power
Condition	6	33510936.073	5585156.012	332.791	<0.0001	1996.747	1.000
Residual	63	1057314.241	16782.766				

Table 2. Means table for 10E4 cells ml^{-1} (effect:condition)

	Count	Mean	SD	SE
PC	5	2805.234	434.068	194.121
NC	5	8.700	4.060	1.816
SAL	12	82.729	7.044	2.033
HBSS	12	99.292	12.043	3.476
MLK	12	97.083	9.533	2.752
P50	12	216.312	82.879	23.925
P100	12	204.854	142.984	41.276

PC, positive control; NC, negative control; SAL, saline; HBSS, Hank's Balanced Salt Solution; MLK, milk; P50, Propolis 50%; P100, Propolis 100%.

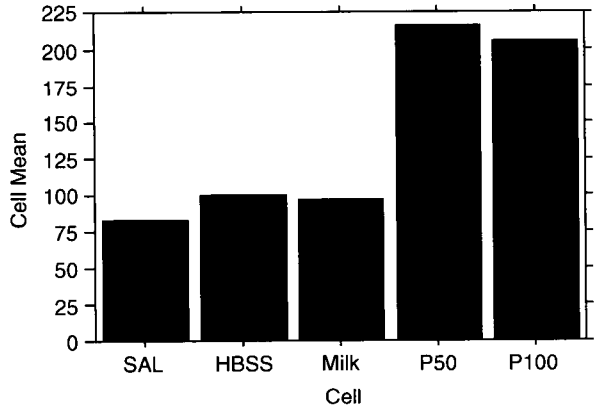

Fig. 1. Interaction bar plot for 10E4 cells ml^{-1} (effect:condition).

and SD of all groups. The teeth stored in Propolis 50% and Propolis 100% demonstrated the highest number of viable PDL cells followed in rank order by HBSS, milk, and saline. A post hoc Fisher's PLSD test showed that both Propolis groups demonstrated a significantly higher number of viable PDL cells than HBSS, milk, or saline. There was no significant difference between Propolis 50% and Propolis 100%. There was also no significant difference in the number of viable PDL cells between HBSS, milk, and saline (Fig. 1). All experimental solution groups were significantly lower than positive controls.

Discussion

Hammer (12) first addressed the importance of PDL cell viability prior to replantation when he demonstrated that the length of survival of a replanted tooth is directly correlated with the amount of viable periodontal membrane. Since that time, scientific inquiries have been done with the hope of finding the optimum set of circumstances that will keep the highest quantity of PDL cells alive postreplantation. Researchers have questioned what the critical extraoral dry times are for PDL cell viability, as well as which storage media should be used on the avulsed tooth in an attempt to prevent the unpredictable sequelae of inflammatory root resorption or replacement resorption postreplantation.

Many studies have examined the critical dry times before irreparable damage to the PDL cells has occurred. Andreasen & Hjørting-Hansen (13) showed that teeth replanted within 30 min had a better success rate than those that were extraoral for longer periods of time before replantation. Other investigators have shown that at 2-h dry time, no vital PDL cells remain (14, 15). In the current investigation, a 30-min dry time was chosen, as 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 min represents a typical clinical scenario during which the avulsed tooth may remain dry before being placed into a storage media.

Numerous studies have tried to assess the optimum storage media for PDL cell viability and preservation (16–18). Cvek et al. (19) found that avulsed teeth soaked in an isotonic saline for 30 min before replantation showed less resorption than those stored dry between 15 and 40 min. Lindskog et al. (20) in an *in vivo* study with monkeys compared saliva with milk and concluded that saliva was less suitable than milk because of its low osmolality and higher risk for bacterial contamination. Matsson et al. (21) examined extracted dog's teeth stored dry for 15, 30, or 60 min and then replanted, versus teeth stored dry for the same dry times but also soaked in HBSS for 30 min before replantation. These researchers found significantly less resorption in the HBSS-soaked teeth for all dry times. Hiltz & Trope (22) found that human lip fibroblasts retained the most viability at 168 h in Viaspan versus HBSS, which kept 76% of the fibroblasts vital for 96 h. They also showed at 6 h that milk was comparable to HBSS or Viaspan.

In terms of storage time periods, past studies have used time frames of 30 min (19, 20) or 45 min (10, 23). The current study stored the teeth in the experimental storage solution for 45 min. This time period was chosen as it allows for comparison with previous investigations.

In the dental literature, various techniques have been used to quantitate the number of viable PDL cells. Reinholdt et al. (24) used a stepwise trypsinization procedure by exposing samples to trypsin three consecutive times for 20 min each. Soder et al. (15) utilized chromogenic stain to quantitate viable PDL cells. Patil et al. (8) used a stepwise trypsinization procedure and fluorescein diacetate as a new staining technique for determining the viability of PDL cells in simulated avulsion injuries.

In the current study, to minimize the exposure of cells to active trypsin and to preserve maximum cell viability, the root surface was treated with collagenase and dispase grade II as was performed in the work by Pileggi et al. (10). This procedure allowed rapid

cell retrieval and maintained maximum cellular integrity, as was demonstrated by the positive control samples.

Propolis, an antibacterial and anti-inflammatory resinous bee-hive product, has never been tested for its potential benefits on PDL cells of an avulsed tooth. This study compared two different concentrations of Propolis to HBSS, saline, and milk in terms of PDL cell viability. Both Propolis groups demonstrated significantly more viable PDL cells than either HBSS, milk, or saline. The Propolis 100% samples were not significantly different from Propolis 50% samples, indicating that the former concentration was no more toxic to the PDL cells. HBSS, milk, and saline were not significantly different, and this result is in agreement with a previous investigator's study (10).

As with many *in vitro* studies, limitations and variabilities often exist. In this investigation, the patient population from which the extracted teeth were obtained had an average age of 45 years. It's likely that a younger patient would have had better healing capabilities, and possibly more PDL cells would have remained viable. Two oral surgery residents performed the extractions, which may have introduced variable trauma during the actual extraction; this could have added to the variability of PDL cell viability counts. While Trypan Blue testing has been used to assess cell viability in many studies, this stain only assesses vitality of the cell and not the actual physiologic health or metabolic capabilities of the cell. The health status of a viable PDL cell is likely critical to prevention of resorptive sequelae postreplantation. Two observers participated in counting the viable PDL cells, which adds to the variability and possibility of human error in the counting process.

Despite the *in vitro* limitations and variabilities encountered in this study, Propolis demonstrated promising results in terms of maintaining PDL cell viability. As with most substances used for 'natural' therapies, there is no standard recommended weight per volume of solution. Furthermore, research shows Propolis as an aqueous extract as well as an ethanol extract, with the latter being the more common formulation (25). A review paper by Burdock (25) concluded that Propolis is relatively non-toxic, with a no-effect level of 1440 mg kg^{-1} day^{-1} in mice according to the result obtained in a 90-day mouse study performed by Hollands et al. (26).

As Propolis could be beneficial to avulsed and replanted teeth, further research is needed to determine a standard formulation for therapeutic use. Moreover, further *in vitro* and *in vivo* studies should be conducted with variable dry times and longer storage times to determine if there is a certain threshold concentration that may be toxic to cells. To date, the trauma literature has not put forth a storage media for the avulsed tooth that not only keeps PDL cells alive but also has antibacterial and anti-inflammatory abilities. Along with Propolis's potential to keep PDL cells viable, perhaps future research can demonstrate how its antibacterial and anti-inflammatory properties may be effective in preventing the resorptive sequelae that often lead to loss of the tooth postreplantation.

Conclusions

1 Within the parameters of this study, it appears that Propolis may be able to maintain PDL cell viability better than HBSS, milk, or saline.
2 The collagenase and dispase assay appears to be a viable method for evaluating PDL cell viability.

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