Evaluation of Periodontal Ligament Cell Viability in Honey as a Storage Media at Different Time Intervals: An In Vitro Study

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Abstract

Aim: To evaluate the viability of human periodontal ligament (PDL) cells in honey when used as a storage media at different time intervals.

Materials and methods: Fifty freshly extracted human premolars were divided into four experimental groups: group I: stored in honey immediately after extraction; group II: stored in honey after 30 minutes extraoral dry time; group III: in Hank’s balanced salt solution (HBSS) immediately (positive control); and group IV: bench-dried for 8 hours with no media (negative control). Groups I, II and III were further divided into three subgroups, to test viability at different time intervals of 3, 6, and 24 hours. The PDL tissue derived was subjected to the trypan blue dye exclusion test. The number of viable cells was estimated with a hemocytometer and the data were statistically analyzed, and the level of significance was kept at p < 0.05.

Results: Groups I and III showed no statistically significant difference in percentage of viable PDL cells after 3 hours (p = 0.339) and 6 hours (p = 0.142), and group II had a statistically lower percentage of viable cells compared to groups I and III (p < 0.001). After 24 hours, group III had highest percentage of viable cells. Group IV had a consistently lower percentage of viable cells.

Conclusion: Within the limitations of this study, it appears that honey may be as efficient as HBSS for storage of avulsed teeth up to 6 hours.

Clinical significance: Commercial honey meets most requirements of an ideal storage media. Being more readily available, inexpensive, and having several therapeutic properties can make it a popular storage media for short duration of storage.

Keywords: Avulsion, Cell viability, Dental trauma, Honey, Storage media.

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Introduction

The International Association of Dental Traumatology identifies avulsion as a dental emergency accounting for 0.5–16% of all injuries to the teeth. Avulsion or exarticulation has been defined by the World Health Organization’s (WHO) adaptation of Andreasen’s Classification as “total displacement of a tooth out of its alveolar socket.” The most commonly affected teeth are the maxillary central incisors. Avulsion injuries most often tend to occur in children between 7 years and 9 years of age, at the time the permanent incisors are in an active stage of eruption.¹ Since most avulsions occur before the patient’s facial growth is complete, maintaining the tooth and its supporting alveolar bone is critical until completion of facial growth.²

Replantation is the treatment of choice in most cases of an avulsed tooth. However, in all cases, immediate replantation may not be feasible.³ Immediate intervention for emergency management is essential to the prognosis of a replanted tooth and this includes the maintenance of viability of periodontal ligament (PDL) cells on the root surface, an uninterrupted root cementum, and minimal microbial contamination, all of which depend on factors such as the extraoral dry time, the type of storage media used for transporting the avulsed tooth, and any alterations in the root surface.⁴

An ideal storage medium is one that is capable of maintaining PDL cell viability alongside presenting clonogenic capacity, antioxidant property, no or minimal microbial contamination, ready accessibility, and has a low cost.⁵ So far, Hank’s balanced salt solution (HBSS) has been considered the gold standard for transport of an avulsed tooth.⁶ Various other types of wet storage media have been investigated, but practicalities of their use such as limited shelf life, high cost, and, most importantly, lack of availability at the site of accident make most of them less ideal.⁶

The literature has shown honey to have broad-spectrum antibacterial properties, typically against microorganisms infecting contaminated wounds. It has potent wound-healing properties, a high antioxidant capacity, is easily available, and has a long shelf-life, fulfilling most of the ideal requirements of a storage media.⁶ Other products sourced from bees such as honey-milk and propolis, having similar properties, have been successfully used as storage media for extraoral dry times up to 6 hours,⁸ but there is lack of awareness, availability, and accessibility for most of the population, which makes them less ideal.
Honey as a Storage Media for Avulsed Teeth

Honey, on the other hand, is routinely used in households for domestic purposes. It can be locally sourced or bought over-the-counter, increasing its accessibility. It has been used for wound management and has advantages over conventional medicines in these scenarios. The ability of commercially available honey to maintain PDL cell viability has not yet been documented in the literature. Thus, this study aims to evaluate the viability of human PDL cells in honey as available commercially, when used as a storage media at different time intervals.

Materials and Methods
Standardization of Storage Media Used
The pH of honey varies depending upon its source and the amount of processing it undergoes. Thus, to standardize the honey used in this study, four brands of commercially available honey were tested for their pH with a digital pH meter. These included Patanjali Honey (Patanjali Consumer Products Private Limited, Greater Noida, Uttar Pradesh, India), Dabur Honey (Dabur India Limited, Solan, Himachal Pradesh, India), Organic Tattva Himalayan Forest Honey (Mehrotra Consumer Products Private Limited, Greater Noida, Uttar Pradesh, India), and Apis Himalayan Honey (Apis India Limited, Roorkee, Uttarakhand, India). Dabur Honey had the highest pH of 4.8 among the selected brands and was selected as storage media for this study. Any further mention of honey in the methodology is pertaining to this brand.

Sample Collection
This study was conducted after obtaining ethical clearance from the ethics committee of the institution. The study was conducted in A. B. Shetty Memorial Institute of Dental Sciences, NITTE (Deemed to be University), Mangaluru. Isolation of periodontal ligament cells was done from 50 human premolars extracted for orthodontic purposes after obtaining each patient’s consent. Each tooth was held at the coronal region, and 3 mm of PDL over the root surface was scraped from the cervical margin with the help of a No. 15 blade to remove cells that might have been damaged during extraction.

The teeth were randomly divided into four groups and transferred to the respective storage media as follows: (1) group I—immediately in honey (n = 15), (2) group II—in honey after an extraoral dry time of 30 minutes (n = 15), (3) group III—immediately in HBSS (Save-A-Tooth system, Phoenix-Lazerus, Inc. Pottstown, PA, USA), which formed the positive control (n = 15), and (4) group IV—left to bench dry for 8 hours without the use of any storage media, which formed the negative control (n = 5). Groups I, II, and III were further divided into three subgroups of five teeth each, to be maintained in the respective storage media at different intervals of 3, 6, and 24 hours.

Cell Culture
The teeth were transported in sterile Falcon’s tubes containing the respective media to NITTE University Centre for Stem Cells and Regenerative Medicine (NUCSRem), K. S. Hegde Medical Academy, NITTE (Deemed to be University) for processing. At the end of the immersion time of the respective groups, the individual teeth were cleansed with phosphate buffered saline (PBS). Periodontal ligament was separated from the teeth using a sterile surgical blade No. 21. The scraped tissue was transferred to a 1-mL microcentrifuge tube with 0.5 mL PBS and centrifuged for 5 minutes at 2,000 rpm to pelletize the PDL tissue, after which the PBS was removed. To the PDL tissue pellet, 0.5 mL of 0.1% collagenase type I enzyme (Gibco, Invitrogen) was added and incubated for 1½ hours. Once the tissue was completely digested, the cells were pelletized by centrifuging them at 1,200 rpm for 5 minutes at room temperature. The supernatant was discarded with sterile micropipettes.

Trypan Blue Dye Exclusion Test to Assess Viability of PDL Cells
The cell viability was measured by the trypan blue dye exclusion test. Cell suspension of volume 100 μL was taken in a fresh 1.5-mL microcentrifuged tube and to that 100 μL of 0.4% trypan blue dye was added. The contents were mixed gently and incubated at room temperature for 5 minutes and then loaded in a Neubauer hemocytometer to count the number of cells. Trypan blue is a vital stain that stains the nonvital cells blue in color and the vital cells appear colorless or transparent. The number of vital and nonvital cells was calculated against the total number of cells at 10x magnification under a light microscope. The total number of viable cells was counted in four different fields and the mean was expressed in percentage. Images of vital and nonvital cells as detected under the microscope after trypan blue staining are depicted in Figure 1 for group I, Figure 2 for group II, and Figure 3 for group III.

Statistical Analysis
Descriptive and analytical statistics were done. The one-way analysis of variance (ANOVA) test was used to check mean differences among the groups. Intergroup comparisons were made using the post hoc Tukey’s test. Intragroup comparisons were done using the paired sample t-test. The level of statistical significance was kept at p < 0.05.

Results
The mean values of viable PDL cells at different time intervals are tabulated as shown in Table 1. Hank’s balanced salt solution (group III) displayed the highest viable cells at all three storage time periods (p < 0.05), i.e., 98.89 ± 1.07 at 3 hours, 96.09 ± 1.68 at 6 hours, and 92.77 ± 2.60 at 24 hours. The mean viable cells of teeth placed immediately in honey (group I) were 96.43 ± 3.83 at 3 hours, 90.02 ± 6.80 at 6 hours, and 84.15 ± 3.51 at 24 hours. After 3 and 6 hours of storage, the percentage of mean viable cells was comparable to those of the positive control (group III). Group II, with
an extraoral dry time of 30 minutes had a statistically significant difference in percentage of viable PDL cells compared with groups I and III with mean values of 84.76 ± 2.00 at 3 hours, 63.13 ± 4.56 at 6 hours, and 27.45 ± 7.07 at 24 hours. The negative control (group IV) showed consistently lowest percentage of viable cells, i.e., 14.16 ± 0.35. The intergroup comparison is shown in Table 2.

Intragroup comparisons among the three experimental groups—groups I, II, and III—are depicted in Figure 4. There was no statistically significant difference (p = 0.080) between the

Table 1: Mean viable cells at different time intervals expressed as percentage

|       | 3 hours  | 6 hours  | 24 hours |
|-------|----------|----------|----------|
| Group I | 96.43 ± 3.83 | 90.02 ± 6.80 | 84.15 ± 3.51 |
| Group II | 84.76 ± 2.00 | 63.13 ± 4.56 | 27.45 ± 7.07 |
| Group III | 98.89 ± 1.07 | 96.09 ± 1.68 | 92.77 ± 2.60 |
| Group IV | 14.16 ± 0.35 | 14.16 ± 0.35 | 14.16 ± 0.35 |

Table 2: Post hoc pairwise intergroup comparison of viability of PDL cells at different time intervals

|                  | 3 hours  | 6 hours  | 24 hours |
|------------------|----------|----------|----------|
| Group I vs group II | Statistically not significantb | Statistically not significantc | Statistically not signifi cand |
| Group I vs group III | Statistically significanta | Statistically significanta | Statistically significanta |
| Group II vs group III | Statistically significanta | Statistically significanta | Statistically significanta |
| Group II vs group IV | Statistically significanta | Statistically significanta | Statistically significanta |
| Group III vs group IV | Statistically significanta | Statistically significanta | Statistically significanta |

* p < 0.001
* p = 0.339
* p = 0.142
* p = 0.022

percentage of viable cells in group I at 3 and 6 hours of storage time. However, the mean viable PDL cells reduced significantly from 3 to 24 hours (p = 0.001) and 6 to 24 hours (p = 0.025) in viable cells. Group II showed a steep decline, which was statistically significant (p < 0.001). Group III showed no statistically significant difference (p < 0.05) at all three storage time periods.

Discussion

A common sequela following replantation is replacement resorption, which eventually remodels to ankylosis, or loss of the tooth. Vascular supply is lost to the pulp and periodontal ligament when the tooth is avulsed. The survival rate of the replanted tooth is directly dependent on the viability of PDL cells. For this, exogenous supply of nutrients is essential, necessitating storage of the avulsed tooth in a suitable medium, to preserve the viability of PDL cells on the root surface. Several experimental studies have indicated that the type of storage media is more crucial to the viability of PDL cells than the extra-alveolar dry time, while determining the prognosis.
of replanted tooth. Selection of an appropriate storage media has shown success even after extended extra-alveolar periods of several hours.1

Human PDL fibroblasts are responsible for rapid periodontal healing and root surface repair. Integrity of these cells is imperative to the success of replantation of the tooth.11 Fibroblasts obtained from the human PDL can accurately reflect the potential to remain viable in a storage medium, rather than those obtained from alternative sources.12 Hence, in this study, we decided to evaluate the viability of human PDL cells while using honey as a storage media.

Honey has been used traditionally by Egyptians, Greeks, Romans, Chinese, and Indians to heal wounds and various ailments including gastric ulcers, aphthous ulcers, and persistent cough. The active components of honey are glucose, fructose, flavonoids, polyphenols, and organic acids. Other constituents such as ascorbic acids, proteins, carotenoids, and enzymes, including glucose oxidase and catalase, along with the active components contribute to the properties responsible for health benefits of honey.10 It exhibits diverse therapeutic properties such as broad-spectrum antibiotic, anti-inflammatory, antimutagenic, and anticarcinogenic properties, as well as expedites wound healing.13 Clinical evidence also supports the specificity, sensitivity, and effectiveness of honey in the management of wounds and it has been illustrated that honey is superior to the conventional and modern wound dressings.10 As mentioned above, several characteristics of honey fulfill most of the ideal requirements of a storage media. Hence, it was decided to evaluate the feasibility of using commercially available honey as a storage medium.

The pH value of the storage medium is essential for optimal cellular growth. It is most favorable in media with a pH ranging between 7.2 and 7.4, a wider range being 6.6–7.8.14,15 This made it necessary to evaluate the pH of honey used for this study. Naturally occurring honey has a lower pH and thus may not be suitable for survival of the cells. Its availability is questionable in clinical scenarios and since there are several types of naturally acquired honey, the contents of each cannot be standardized. Commercially available honey on the other hand is standardized, easily available, and has a long shelf life of up to 5 years, and thus Dabur Honey was chosen to be tested as a storage medium in this study. The pH of the commercially available honey products has not been documented and therefore testing the same was essential, before use as a storage media in this study.

While the type of storage media used for preservation of viable PDL cells is crucial to the survival of the replanted tooth, another important prognostic factor is the extraoral dry time, i.e., the time elapsed from the avulsion of the tooth from the alveolar socket to the placement of the tooth in any transport media.16 According to Andreasen and Hjørring-Hasen, a better prognosis was observed when teeth were replanted within 30 minutes of extraoral dry time, as compared to those replanted after extended periods of extraoral dryness before replantation.17 After 30 minutes of extraoral dry time, the undifferentiated mesenchymal cells lose their ability to differentiate into fibroblasts necessary for repair.2 Thereby, 30 minutes can be considered as the critical time after which damage to the PDL cells is hastened.18 Therefore, it was decided to assess the efficacy of honey at different time intervals of 3, 6, and 24 hours when placed immediately in honey, and after 30 minutes of extraoral dry time.

Hank’s balanced salt solution, which is considered the gold standard for storage media,19 comprises of sodium chloride, D-glucose, potassium chloride, sodium bicarbonate, monobasic potassium phosphate, calcium chloride, and magnesium sulfate anhydrous. It has a pH of 7.4 and osmolality of 280 mOsm/kg.20 Hank’s balanced salt solution possesses optimal properties for the maintenance of PDL cell viability. A study by Hiltz and Trope concluded that after 96 hours’ storage in HBSS, 70% fibroblasts retained their vitality.21 Based on the past literature, HBSS was selected as the positive control for this study.

Dry storage media, due to reasons stated above, is the least preferred form of storage for avulsed teeth.22 The negative control (group IV) in this study thus comprised of teeth left bench-dried for 8 hours without the use of any storage media.

The PDL cell viability has been routinely assessed by in vitro assays owing to their relatively simple and reproducible methodology, where the independent and dependent variables can be well controlled.12 The 0.4% trypan blue exclusion staining technique was carried out to assess the viability of cells. The negatively charged chromophore on the cell membrane does not take up the stain and helps differentiate the viable cells from the nonviable. Any damage to the cell membrane will interfere with the charge and thus easily take up the color of the stain, confirming nonviability of the cell. This method was chosen on account of it being simple and convenient to use.23

The pH of honey used in this study was 4.8, lower than the range required for cellular proliferation. However, cell viability was still comparable to HBSS. This could be attributed to the rich nutrient composition and high levels of antioxidants present in honey. The free radicals accumulated due to the inflammatory response produced when the tooth is avulsed prevents cell proliferation. The antioxidative property of honey aids in removal of these free radicals, providing protection against lipid peroxidation of the cell membrane, thereby stimulating the growth of epithelial cells, which play a crucial role in the healing process and help in repair of damaged tissue.24 The low pH could also be responsible for an increased antibacterial efficacy of this medium.

The application of honey can accelerate the healing process, bring about wound cleansing, decrease or eliminate infection, minimize inflammation, and augment tissue regeneration.10 Hammond et al. in 2013 concluded from their study that honey has a strong antibacterial action against Clostridium difficile, a common organism found in wounds contaminated by soil.25 Honey-impregnated gauze has been used to successfully cleanse extraction sockets to reduce postoperative inflammation and complications by Soni et al. in 2016, indicating its potent wound-healing properties.26 Thus, honey could also aid in healing the socket of the avulsed tooth after replantation of the tooth that is stored in it.

According to the results of this study, the efficacy of honey was comparable to that of HBSS, when the tooth was immediately transported to the media, and stored for up to 6 hours. A study by Nozari et. al. in 201321 evaluated the efficacy of long-shelf-life honey-milk and demonstrated efficacy comparable to HBSS up to 9 hours. Honey-milk may not be easily accessible at the site of injury and thus honey could be a good alternative.

Propolis has been the most studied plant-derived storage media according to a recent systematic review.28 However, it is available in varying concentrations and till date, there is no consensus on its standardization for maximum efficacy as a storage media. This natural product is also not as easily available and accessible at the time of emergency as opposed to easily and commercially available honey products.
When the tooth was stored in honey, after a dry time of 30 minutes, 84.76% of cells were still viable after 3 hours. After 6 and 24 hours, this group showed a decreased percentage of viable PDL cells. For storage in HBSS, 84.76% of cells were viable after 3 hours. After 6 and 24 hours, this group showed a decreased percentage of viable PDL cells. Therefore, honey can be recommended as an ideal storage media for an avulsed tooth.

This study has certain limitations. The sample size of each group studied was limited. To increase the power of the study, a larger sample size is recommended. The PDL tissue excision from each tooth was performed by the same investigator. This minimizes any gross variation in the amount of tissue excised. However, the tissue was not quantified before analysis to rule out bias due to varying volume of tissue collected.

Further in vitro investigations may be carried out to validate the results obtained in this study with a larger sample size. In vivo studies would provide further insight for the same.

Conclusion

Although the gold standard of storage media for avulsed teeth has been identified as HBSS, it has drawbacks as mentioned earlier. Thus, there is a need for a more practical and equally effective medium. Honey, a natural therapeutic product that is easily available worldwide, has a multitude of remedial properties making it a good alternative for storage media. Considering all of the above, we can conclude that honey is comparable to HBSS and can be used as a storage media for up to 6 hours of storage, depending on the extraoral dry time of an avulsed tooth.

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