Original Article

Comparative Evaluation of Behaviors of Three Naturally Occurring Products, Namely Propolis, Milk, and Egg Albumin When Used as Storage Media in Extracted Teeth for Orthodontic Purpose

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Abstract

Background: The reasonably successful prognosis in retaining avulsed tooth in its respective position depends on the maintenance of the viability of the periodontal ligament (PDL) cells during the replantation procedure. Various synthetic media have been tried for preserving the viability of PDL cells with considerable success. However, easy availability of these media had been a problem at various occasions. Hence, an attempt was made to test the properties of easily available natural products such as Propolis, milk, and egg albumin. **Aim**: This study was aimed at comparing the behaviors of three naturally available media, namely propolis, milk, and egg albumin when used as the storage media in preserving the viability of traumatized periodontal cells in an avulsed tooth. **Materials and Methods:** A total of 50 premolars with closed root apices indicated for the extractions for orthodontic treatment were selected. Initially, these teeth in the experimental group were stored dry immediately after the extraction for 30 minutes, and then, immersed in the respective storage media for 45 minutes. The teeth in the positive control group were assessed immediately after the extraction, whereas the negative control teeth were bench dried for 8 hrs. All the five group samples were then incubated with collagenase and phosphate-buffered saline for 30 minutes, centrifuged and labeled with 0.5% trypan blue for the determination of cell viability. The cells were counted under the light microscope. The statistical analysis was carried out by applying the unpaired *t*-test. **Results:** No statistically significant difference was observed in viable PDL cell counts when compared among propolis, milk, and egg albumin. **Conclusions:** Propolis, milk, or egg albumin could be a good naturally available storage media for avulsed teeth.

Keywords: Avulsion, cell viability, storage media, propolis

Introduction

Dental trauma is extremely common among children. Andreasen and Andreasen (1990) predicted that the incidence of these injuries may eventually surpass the incidence of dental caries.[1] Tooth avulsion is the complete displacement of a tooth from its socket due to accidental or nonaccidental injuries.[2] If the avulsed tooth is replanted immediately within 5 min yields best result, but if it is not possible, avulsed tooth must be stored in the storage media to keep periodontal tissue under good state until replantation.[3] A storage medium is defined as a physiological solution that closely replicates the oral environment to help preserve the viability of periodontal ligament (PDL) cells following avulsion.[4] Various extra-Alveolar storage media such as Hank's Balanced salt solution, Viaspan, Custodiol, Eagle's Minimum Essential Medium, saline, natural products

Access this article online

Quick Response Code:

Website: www.archtrauma.com

DOI: 10.4103/atr.atr_16_20

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How to cite this article: Shingare P, Chaugule V. Comparative evaluation of behaviors of three naturally occurring products, namely propolis, milk, and egg albumin when used as storage media in extracted teeth for orthodontic purpose. Arch Trauma Res 2020;9:129-34.

Received: 18-03-2020, **Accepted:** 30-05-2020, **Published:** 22-08-2020

such as water, saliva, milk and its variations, propolis, green tea, Morusrubra (red mulberry), egg white, coconut water; rehydrating solutions like Gatorade and Ricetral, and even contact lens solutions have been tried with variable success in case of intentional or accidental replant procedures.[4]

In cases of tooth avulsion preservation of viability and vitality of PDL cells attached to the root surface for effective reattachment of PDLs has always remained as mainstay of replantation procedure. Extraoral time and storage medium play a vital role in this.[5,6] Studies have shown that an avulsed tooth can be replanted without complications within 1–3 h of being placed in suitable storage conditions.[6] An ideal storage medium is the one which is capable of preserving the viability, mitogenicity, and clonogenic capacity of the damaged PDL cells to facilitate the repopulation of the denuded root surface, thereby preventing further root resorption.[6,7] The storage media should also have physiological osmolality and pH and should be maintained at an appropriate temperature to allow optimal cell growth. Finally, the ideal storage media should be readily available for use in emergency situations.[8]

Synthetic media such as HBSS, Viaspan have been proved to be the best, but in India and in any other developing countries, they may not be readily available at the time of emergency in that region when it is required. Hence, the naturally occurring media which would be available easily at the time of accident should be searched. Hence, we selected three such media such as milk, egg albumin, and propolis to study the viability of PDL cells.

Blomlof *et al*. suggested that the osmolality of milk being within physiologic limits and markedly contains fewer bacteria makes it more suitable storage medium.[9,10] Egg albumin is another naturally occurring media which can be made easily available at an emergency situation. It has a high amount of protein (albumen), vitamins, water, and additional lack of microbial contamination.[10,11] Propolis also known as Russian penicillin is a sticky resinous material that honey bees collect from certain plants and flowers. It has a high medicinal value due to its inherent rich source of bioflavonoid, iron, and zinc from within and has antimicrobial, anti-inflammatory properties[12,13]. The purpose of this study was to compare egg albumin, milk, and propolis for their ability to maintain periodontal cell viability *in vitro*.

Materials and Methods

This experimental *in vitro* study was carried out in the department of Pediatric and Preventive Dentistry, to assess the viability of the PDL cells in propolis, milk, and egg albumin after discussing and getting the approval from the ethics committee.

A total number of 50 non carious premolars with closed apices, healthy periodontium, and indicated for orthodontic extraction were selected for the study after having taken consent from patient and Institutional Ethics Committee. The extractions were carried out as much atraumatically as possible with minimum damage to the PDL membrane. After extraction, the teeth were held with forceps in the coronal region and the 3 mm of PDL from the cervical root surface was scraped using BP blade number 15 to remove the cells that might have been damaged during extraction.[14,15] The teeth were then randomly divided into three experimental groups (propolis, milk, and egg albumin) and two control groups (positive and negative control) containing $n = 10$ samples in each group namely,

- Group 1 – Positive control (+ve control)
- Group 2 – Propolis
- Group 3 – Milk
- Group 4 – Egg albumin
- Group 5 – Negative control (−ve control)

The propolis was procured from C C Pollen co. Arizona. (HIGH DESERT^R Bee Propolis extract 33%). The milk used was pasteurized and homogenized cow milk from Govind, a locally available brand. A freshly teased out egg white from egg yolk was stored in the separate bottle for each sample.

The teeth in the experimental group were stored dry for 30 min followed by 45 min immersion in one of the three experimental media. Each tooth in all groups was treated separately and incubated for 30 min in 15 ml falcon tubes with 2.5 ml solution of 0.2 μg/ml of collagenase in phosphate-buffered saline. Whilst the teeth in the positive control group after extraction were immediately treated with collagenase (Himedia co) without drying and storing. The teeth in the negative control group were bench dried for 8 h with no follow-up storage time which was then followed by the treatment with collagenase.

The specimens were then centrifuged for 5 min at 800 rpm and the cells labeled with 0.5% trypan blue (Himedia co.) for the determination of viability.[15] The number of viable and nonviable PDL cells was counted under light microscopy with a hemocytometer, and the data were statistically analyzed.

Results

Table 1 presents the number of viable and nonviable cell counts in all groups. Table 2 presents the mean and standard deviation of all groups. Positive control group showed mean viable cell count of 6.835 and mean nonviable cell count of 0.3650, whereas negative control with bench drying showed only 0.110 mean viable cell count and 2.930 mean nonviable cell count. Table 3 exhibits the variations in viable and nonviable cells between the experimental and control groups. The results of the study showed that the teeth stored in Propolis demonstrated the highest number of viable PDL cells followed in order by milk and egg albumin. Although the difference in three groups was not statistically significant [Table 4 and Graph 1], Graph 2 shows the comparison of the experimental groups with the control groups in viable cell count.

Discussion

After tooth avulsion the PDL tissues begin to dehydrate, so to prevent the damage due to dehydration, prompt replantation

Table 1: Viable and nonviable cell counts in all groups

Serial number	Group 1 +ve control		Group 2 propolis		Group 3 milk		Group 4 Egg albumen		Group 5 -ve control	
	Viable	Nonviable	viable	Nonviable	Viable	Nonviable	Viable	Nonviable	Viable	Nonviable
Cell count $n\times10^5$ Cells/ml										
1	6.25	0.25	4.6	0.25	1.25	0.8	0.95	0.3	0.35	3
2	7.8	0.65	2.7	0.85	1.95	0.65	0.5	0.55	0.1	5.05
3	10.15	0.45	0.6	0.2	4	0.6	0.75	0.6	0.1	4.75
4	4.55	0.5	1.25	0.35	3.1	0.3	2.45	1	0.15	2
5	6.5	0.35	1.9	0.45	0.85	0.25	3.4	2	0.2	0.8
6	5.8	0.11	1.25	0.75	2.9	1	3.5	0.5	0.5	1.5
7	4.7	0.32	4.05	0.55	3.45	1.1	1.4	1.95	0.1	2.8
8	8.3	0.56	3.95	1.05	4.15	0.5	1.75	2.55	0.05	3.95
9	5.2	0.18	2.4	0.95	1.05	0.45	2.05	1.4	0.15	4.15
10	9.1	0.28	1.95	0.65	0.55	0.35	2.45	1.15	0	1.3

Table 2: Mean and standard deviations in all groups

Viable cell count				Nonviable cell count			
Groups	*n*	Mean	SD	Groups	*n*	Mean	SD
Propolis	10	2.4650	1.34785	Propolis	10	0.6050	0.29482
Egg albumen	10	1.9200	1.9199	Egg albumen	10	1.2000	0.75939
Milk	10	2.3250	1.35734	Milk	10	0.6000	0.29059
Positive control	10	6.835	1.9199	Positive control	10	0.3650	0.17213
Negative control	10	0.1700	0.14944	Negative control	10	2.9300	1.50779

SD: Standard deviation

Table 3: Test of variability between the groups (ANOVA test)

Groups	*n*	Mean	SD	ANOVA *P*	*P*	Groups	*n*	Mean	SD	ANOVA *F*	*P*
Viable cell count						Nonviable cell count					
Group 2	10	2.4650	1.34785	25.29	<0.001 (HS)	Group 2	10	0.6050	0.29482	16.04	<0.001 (HS)
Group 3	10	2.3250	1.35734			Group 3	10	0.6000	0.29059		
Group 4	10	1.9200	1.04833			Group 4	10	1.2000	0.75939		
Group 1	10	6.8350	1.91993			Group 5	10	2.9300	1.50779		

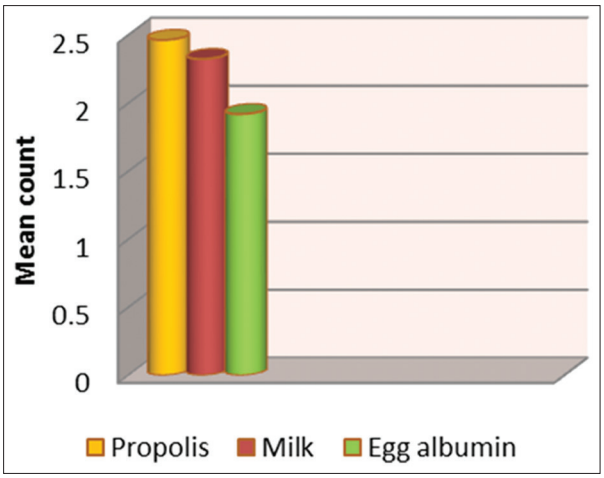
HS: Highly significant, SD: Standard deviation

Table 4: Intergroup comparison of experimental groups

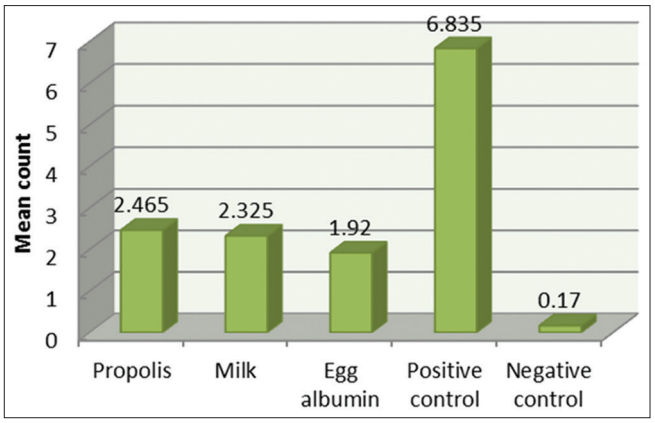
Viable cell count					
Group	*n*	Mean	SD	Unpaired *t*	*P*
Propolis	10	2.4650	1.34785	0.231	0.82 (NS)
Milk	10	2.3250	1.35734		
Propolis	10	2.4650	1.34785	1.009	0.32 (NS)
Egg albumin	10	1.9200	1.04833		
Milk	10	2.3250	1.35734	0.74	0.46 (NS)
Egg albumin	10	1.9200	1.04833		

NS: Not significant, SD: Standard deviation

of the tooth is required. Hammer was the first to demonstrate that length of survival of a replanted tooth was directly correlated with the amount of viable periodontal membrane and this marked the importance of PDL cell viability before replantation. However, immediate replantation may not always be possible. The replantation of teeth beyond 5 minutes of avulsion has been defined by Andresen as delayed replantation. In case of delayed replantation, the avulsed tooth should be stored in appropriate media, which can prevent desiccation and subsequent loss of vitality of PDL.[16] Common failures associated with replanted teeth are external root resorption and pulp necrosis, so a storage medium is desired which can help maintain the viability of PDL cells and prevent the chances of development of external root resorption in future to prevent the failure of the replanted tooth. The storage media for an avulsed tooth should have low bacterial content, physiological osmolarity, a neutral pH, and essential nutrients. HBSS is a widely used standard solution recommended by the International Association of Dental Traumatology as a storage



Graph 1: Comparison of viable cell count in three experimental groups



Graph 2: Comparison of viable cell count in study groups

medium for avulsed teeth. The HBSS is costly and may not be available readily, and hence, there was a need to identify other accessible and affordable storage media.

Literature has shown increased research on naturally occurring storage media such as milk, coconut water, egg albumin, green tea, and propolis is also not an exception to this. Out of which milk, coconut water, and egg albumin can readily be made available at the site of accident or trauma, but this is not so with Propolis. Propolis is now available in the market, but common man may not be aware of it and its usefulness and health benefits. It can be purchased in pharmacies or health food stores. It is available in the form of powder, tablets, capsules, and liquid extracts which can be taken orally. It is available for the topical application in the form of cream, ointment, and spray. Various studies have proved that it has antibacterial, antiviral, antifungal, and anti-inflammatory properties, and hence, it can be considered as a good option for the storage medium.

Different techniques have been used to study the viability of PDL cells following avulsion. Most commonly used technique is cell culture and trypsinization for longer periods of time. The extracellular matrix is composed of collagen and other proteins, and hence, it is reasonable that the use of enzymatic desegregation would provide great number of cells within shorter time frame. Both collagenase and dispase enzymes disrupt the extracellular matrix and cause the release of cells without excessive disruption and destruction of their own membrane. Hence, in our study, we opted for collagenase assay, where in the cells are not subjected to long processing times to determine their viability status. This procedure allowed rapid cell retrieval and maintained cellular integrity[15].

We used the dye exclusion test to determine the number of viable cells present in cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as trypan blue, casin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether the cells take up or exclude dye. Upon examination, a viable cell would show clear cytoplasm, whereas a nonviable cell would exhibit blue cytoplasm. Dye exclusion though is a simple and rapid technique for measuring viable cell counts; cell membrane integrity happens to be a determinant factor. Thus, it would be possible that viability of few cells might have been compromised. Another potential problem is the subjective assessment of dye uptake; hence, small amounts of dye uptake indicative of cell injury might have gone unnoticed. Despite knowing limitations of dye exclusion technique, utilization of 0.5% trypan blue exclusion technique was found to be effective because of its nature of rapidity being performed within 5–10 minutes.[17,18]

In our study, from the mean cell count values, as observed from Table 2, it could be inferred that propolis showed promising results when compared to milk and egg albumin. Increased research on propolis has been seen in the literature in recent years as storage medium for avulsed teeth and as intracanal medicament. Martin and Pileggi showed that propolis could be a better alternative transport medium when compared with HBSS, milk, or saline. They also showed that there was no significant difference between propolis and HBSS in maintaining cell viability.[12,19] In our study, we utilized 33% propolis extract from U. S.A, which is being consumed there as a health drink. Abangari *et al*. compared the number of viable cells at 1 h and 3 h after storage in 10% propolis, 50% propolis, milk, egg white, and HBSS. A significantly more number of viable PDL cells were found in Propolis as compared to other experimental groups.[20] However, Mahal *et al*. reported similar efficacy in propolis (15%), egg albumin, and HBSS.[16,21] Our results are also in accordance with that of Mahal *et al*. showing no significant differences among propolis, milk, and egg albumin. Casaroto *et al*. showed that propolis was better than milk, HBSS, or saline in maintaining PDL cell viability.[22] In the study by Babaji *et al*., Propolis showed more number of viable PDL cells followed by HBSS, *Aloe vera,* and pomegranate.[23] The results of studies on propolis vary because of its use in different concentrations. Variations in the results of researchers could be attributed to the difference in the concentrations used and altered

composition due to plant and flower species from where honey bees collect the honey. All in all, most of the studies have shown positive inclination toward the use of Propolis as a good storage medium.

Milk has been proved to be compatible storage media since long. It is accepted by the American Association of Endodontists as a suitable transport medium for avulsed teeth.[24] Milk prevents cell death, but according to Gamsen *et al*., it does not restore normal morphology and ability to differentiate and mitose.[25] It is able to maintain the osmotic pressure for PDL cells but does not have ability to reconstitute depleted cell metabolites and restore viability. Furthermore, the performance of milk varies with the fat content of the milk. Harkacz *et al*. showed that milk with lower fat content may be more appropriate at maintaining cell viability than milk with higher fat content.[26] Goswami *et al*. showed that it is important to use milk in first 20 min after avulsion.[27] In a study by Velayutham Gopikrishna, it has been shown that the coconut water and propolis maintained more number of viable PDL cells than milk.[12] The results of our study also concur with the results of these studies.

Khademi *et al*.[11] studied the effectiveness of egg albumen and milk as the storage media for avulsed tooth in dogs. The teeth were replanted after storage in the respective storage media, and after 2 months, the sections of the teeth were examined for normal PDL, surface resorption, replacement resorption, or internal resorption. They found that the teeth stored in egg albumen showed the highest incidence of PDL healing while a significantly higher inflammation of PDL in teeth stored in milk for 6 and 10 h. In contrast, they showed extremely high PDL healing after 6 and 10 h in egg albumen. According to Khademi *et al*., the viability of PDL cells in egg albumen was similar to that of HBSS after varying periods of time. However, results of our study are in contrast with that of Khademi *et al*. showing less number of viable PDL cells in the egg albumin group. Rozenfarb *et al*.[28] found no significant difference among Minimum Essential Medium (MEM), egg albumen and milk, similarly our study results too were found to concur with this.

Blomlof *et al*. suggested ideal requirements for a storage medium as osmolarity 290–330 μ osm/L and pH 6.6–7.8.[10] HBSS has been considered as the gold standard among various storage media. It has a balanced pH (7.2) and contains essential metabolites necessary for PDL cell viability. It has been found that it can maintain viability of PDL cells up to 48 hrs.

Common failure associated with replanted teeth is external root resorption. Since propolis has good anti-inflammatory, anti-microbial properties, we selected propolis to investigate whether it could be used as a storage medium and could maintain the viability of PDL cells. If it proved to be good storage medium in terms of maintaining high percentage of viable cells, more *in vivo* research is required to study whether teeth stored in propolis resist the root resorption once replanted in the socket.

In our study, we examined avulsed teeth with 30 minutes of dry time. Further investigations with longer periods of dry time are required to understand the consequences with respect to storage media and PDL cell viability. More *in vivo* research is also required to study a confirm effect on avulsed teeth, when stored in different storage media clinically and radiographically.

Conclusions

1. Within the parameters of this study, we found no significant differences among propolis, milk, and egg albumen when dry out time did not exceed 30 minutes.
2. Trypan blue dye exclusion and collagenase assay appeared to be a viable method for evaluating PDL cell viability.
3. The natural products such as propolis, milk, and egg white could act as appropriate storage media because of their easy availability and potential to maintain the viability of PDL cells for longer durations.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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