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Comparative Evaluation of Different Storage Media at Different Time Intervals in Maintaining Periodontal Ligament Cell Viability- An in Vitro Study

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Abstract

Aim: To evaluate the efficacy of different storage media at different time intervals in maintaining periodontal ligament cell viability.

Material And Methods: One hundred sixty premolars extracted for orthodontic correction were collected from Oral surgery department and segregated equally among eight different storage media, evaluated over stipulated period of time intervals for maintaining viability of PDL cells.

Results: PDL cells viability varied in different storage media over various time intervals of 45 minutes, 2 hours, 6 hours, 24 hours, with Propolis maintained highest PDL cell viability and artificial saliva showed least.

Conclusion: HBSS maintained maximum viable cells for 45 minutes followed with Propolis that showed maximum viable cells even after 24 hours of storage but egg white showed comparable results to both HBSS and Propolis and can be considered a good alternative due to ease of availability and cost effectiveness.

Keywords: Dental trauma, Avulsion, Storage medium, PDL cell viability, Reimplantation, Propolis, HBSS, Egg white

Introduction

Over the past decade, the incidence of dental injury has increased significantly, affecting permanent anterior teeth of children aged 7 to 12 years.[1,2] Children are always interested in exploring their surrounding without knowing

the risks they are exposed to, which can lead to traumatic injuries. Trauma to the teeth can lead to crown or root fracture, luxation injuries or avulsion.[3] The WHO has defined avulsion as the complete displacement of a tooth from its alveolar socket due to traumatic injury.[4] Avulsion is one of the most complex traumatic injury affecting children teeth.[5] It can happen at any age and creates problems for the patient and accounts for 0.5%-16% of traumatic injuries in the permanent dentition, whereas 7-21% in the primary dentition.[6,7] Avulsed tooth replantation depends directly on the viability of periodontal ligament cells, which are influenced by two critical factors i.e the extra-oral dry time and the storage medium in which the tooth is placed. However, the storage medium's ability to support cell viability is more important than the extra oral time to prevent failures of reimplantation such as ankylosis and resorption. [8]

Replanting a tooth within 5 minutes ensures a prompt return to normal function of the PDL differentiation and stem cells.[9] Nevertheless, PDL stem cells are no longer able to differentiate into fibroblasts for more than 15 minutes of dry storage.[10] In addition, all remaining PDL and stem cells on the tooth root are likely to become necrotic after 30 minutes.[11,12] Immediate replantation results in PDL healing up to 85% of mature teeth.[13] The storage media should have a physiological osmolality (290-330 mOsm/kg) and pH (7.2-7.4) and should be maintained at an appropriate temperature to enable optimum growth or survival of cells.[14]

The transportation of the tooth also has a significant impact on the degree of success. The root of the tooth should not be touched and the container used to store avulsed teeth must be unbreakable, non-toxic, leak-proof and easy-to-handle, and sterile.[15] Other contributing factors such as awareness of the correct actions to be taken and the proximity of the scene to a dentist or dental clinic should be taken into account.[16]

Commercial media like Hank's Balanced Salt Solution, and Viaspan are ideally suitable for storage of the avulsed teeth. But the cost and ready availability of these agents at the site of the accident have been the major problems. Other solutions like Propolis, Milk, Saline, Aloevera, Eggwhite, Oral rehydration solution and Artificial saliva have also been used as a storage media. Naturally available storage media such as propolis and aloevera have recently gained popularity in the medicinal field. Propolis has antifungal, antibacterial and anti inflammatory properties and there is presence of bioflavonoids which are the most powerful antioxidant where as Aloevera has antibiotic, Antidiabetic and anticancer properties.

Methodology

Inclusion Criteria

- Non carious Maxillary/Mandibular first or second premolar teeth
- Teeth with normal periodontium and closed apices

Exclusion Criteria

- Teeth with moderate to severe periodontal disease
- Teeth with fractured crown or root
- Associated with pathology

Sample of 160 premolar teeth with normal periodontium and closed apices were collected from department of Oral Surgery had undergone atraumaatic extraction for orthodontic purpose. Coronal 3mm of periodontal ligament was scraped with the curette to remove the cells that have been damaged during extraction of tooth. The teeth were randomly assigned into eight experimental groups for different time intervals i.e 45 minutes, 2, 6 and 24 hours immersion in one of the following experimental storage medias like:

- Group 1- HBSS

- Group 2 - Milk – pasteurized, homogenized milk was use.
- Group 3- Saline
- Group 4- Propolis - Propolis was made into 50% concentration using 0.4% ethanol solution. Propolis 50% was prepared by adding 50 mg ground propolis per 250ml of the 0.4% ethanol solution and before submersion of teeth in propolis, solutions were shaken for 15 minutes.
- Group 5 - Egg white – Egg white was separated from the yolk and collected in a bowl using the half shells.
- Group 6 – Aloe vera- To prepare aloe vera gel, aloe vera leaves were collected and washed. A kitchen knife was used to cut off the outer spiked edges of the leaves. After removing the outer skin of the leaves, the inner gel was extracted and transferred into a blender. The contents were blended thoroughly and filtered through a piece of muslin cloth and were placed into a glass jar with a tight fitting lid.
- Group 7- Artificial saliva
- Group 8- Oral rehydration solution1 teaspoon of ORS powder was taken in 200 ml (one glass) of distilled water and stirred. New experimental solution was made every time

Harvesting of PDL cells: After the stipulated time interval, the teeth were taken to the microbiology laboratory where further procedures were carried out. The teeth were handled by anatomical crown portion during the procedures to prevent damage to the periodontal cells. All the teeth were held with tweezer from the coronal portion and cleansed the root surface twice with sterile isotonic saline to remove the residual storage media.

At different storage time intervals the apical two-third of the root surfaces (as measured from the epithelial attachment) were scraped with No. 15 BP blade in a petridish to obtain the PDL cells. **Fig 1(i)** The scrapings were subsequently added to 15 ml Falcon tubes containing 2.5 ml of phosphate buffer saline. To the above mixture, 0.5mg of Type 1 collegenase (Sigma–Aldrich chemicals) was added, and the mixture was incubated at 37^{o}C for 30 minutes. After incubation, the Falcon tubes were centrifuged for five minutes at 800 rpm.[17]

The supernatant solution was discarded with micropipette, and the centrifuged residue was collected. To this solution, an equal volume of 0.4% Trypan blue stain was added and mixes well. Trypan Blue stains non-viable cells blue and viable cells appeared colorless or pink.[18] After staining, the viable cells appeared colorless or pink. After staining, the viable cells were counted using hemocytometer under an optical microscope**.** [19]**(Fig.1 (ii, iii).**

Determination of the Number of Cells: The viable cells were examined under a microscope at 100x magnification. The numbers of cells were calculated by counting the cells overlying a 4 x 1mm^2 area of the neubauer chamber.[2]

The viable percentage of the cell population of each sample was obtained by:

(UC/TC) X 100 = %

Where, UC- unstained cell count (viable cells), TC total cell count (stained + unstained cells)

Results and Observation

The results as obtained by One Way Anova showed statistically significant difference between the groups at various time intervals.

- HBSS showed maximum viable cells 87% at 45 minutes which further decreased to 73.6% at 2 hours, 60.15% at 6 hours and least 30.4% at 24 hours.
- Propolis as the storage media showed the second best reading of 85.8% viable PDL cells at 45 minutes and further came out best results with increased storage time.
- Eggwhite showed viable cells 83.7% at 45 minutes which further decreased to 70.74% at 2hours, 53.82%

at 6 hours, and least number of viable cells 26.08% at 24 hours.

- Artificial saliva maintained least number of viable cells at 45 minutes, 2 hours, 6 hours and 24 hours storage time intervals than other storage media.

Table 1: ANOVA results for cell viability score among different medium at 45 minutes

Time interval group		Sum of Squares	Df	Mean Square	F	Sig.
45 - minute	Between Groups	6931.860	7	990.266	192.303	.000
	Within Groups	164.784	32	5.149		
	Total	7096.644	39			

Table 2: ANOVA results for cell viability score among different medium at 2 hours

Time interval group		Sum of Squares	df	Mean Square	F	Sig.
2 - hour	Between Groups	7963.670	7	1137.667	483.985	.000
	Within Groups	75.220	32	2.351		
	Total	8038.890	39			

Table 3: ANOVA results for cell viability score among different medium at 6 hours

Time interval group		Sum of Squares	df	Mean Square	F	Sig.
6- hour	Between Groups	8109.924	7	1158.561	328.169	.000
	Within Groups	112.972	32	3.530		
	Total	8222.896	39			

Table 4: ANOVA results for cell viability score among different medium at 24 hours

Time interval group		Sum of Squares	df	Mean Square	F	Sig.
24- hour	Between Groups	7903.882	7	1129.126	610.834	.000
	Within Groups	59.152	32	1.848		
	Total	7963.034	39			

Table 5: Homogenous subsets for 45 minutes time interval

Media used	N	Subset for alpha = 0.05			
		1	2	3	4
Artificial Saliva	5	47.8600			
ORS	5		56.4800		
Saline	5			72.1600	
Aloe Vera	5			74.5600	
Milk	5			74.6000	
Egg White	5				83.7600
Propolis	5				85.8200
HBSS	5				87.0000
Sig.		1.000	1.000	.687	.347

Table 6: Homogenous subsets for 2 hours time interval

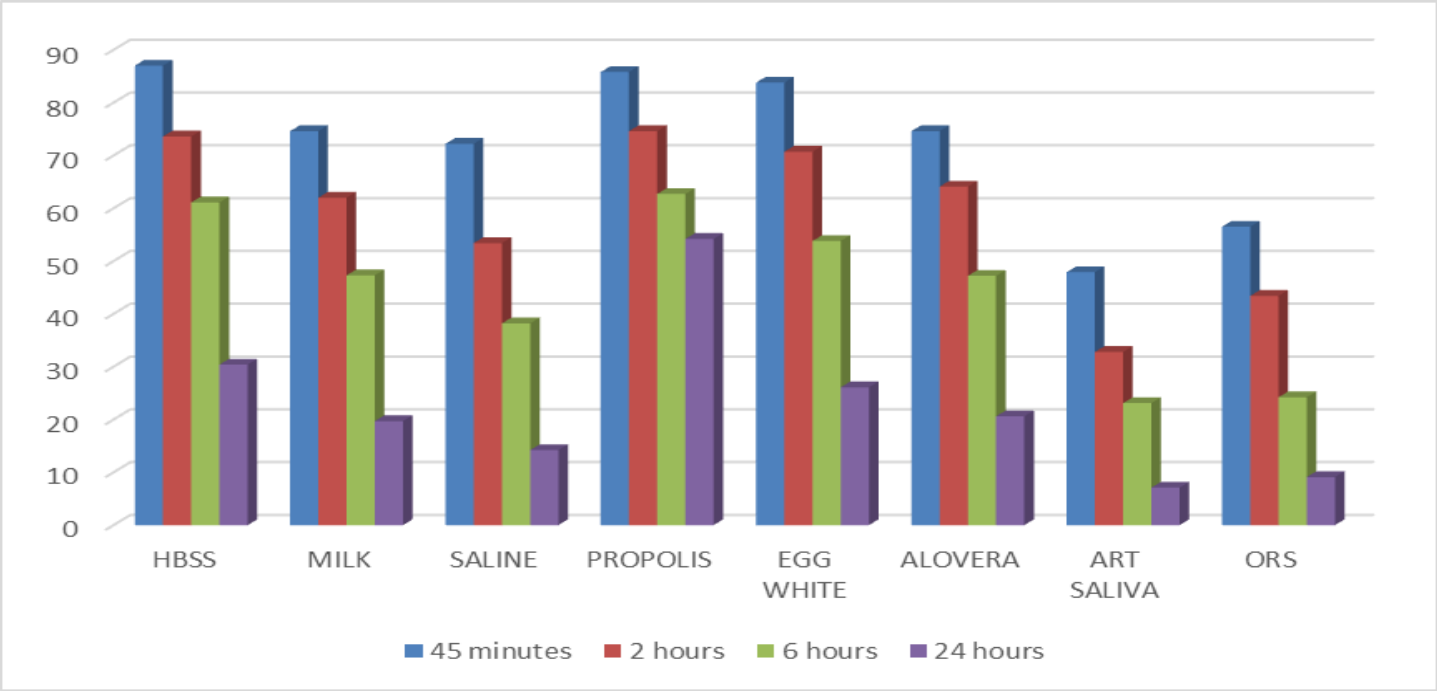
Media used	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Artificial Saliva	5	32.7600					
ORS	5		43.3600				
Saline	5			53.4000			
Milk	5				62.0200		
Aloe Vera	5				64.1200		
Egg White	5					70.7400	
HBSS	5					73.6000	73.6000
Propolis	5						74.5800
Sig.		1.000	1.000	1.000	.398	.096	.969

Table 7: Homogenous subsets for 6 hours time interval

Media used	N	Subset for alpha = 0.05				
		1	2	3	4	5
Artificial Saliva	5	23.0800				
ORS	5	24.1600				
Saline	5		38.1600			
Aloe Vera	5			47.1800		
Milk	5			47.3000		
Egg White	5				53.8200	
HBSS	5					60.1500
Propolis	5					67.1600
Sig.		.983	1.000	1.000	1.000	1.000

Table 8: Homogenous subsets for 24 hours time interval

Medium Used	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Artificial Saliva	5	7.0600					
ORS	5	9.0800					
Saline	5		14.1600				
Milk	5			19.7100			
Aloe Vera	5			20.5600			
Egg White	5				26.0800		
HBSS	5					30.4000	
Propolis	5						54.2200
Sig.		.300	1.000	.973	1.000	1.000	1.000



Graph 1: Comprehensive representation of cell viability scores in different storage medium for different time- interval group.

Discussion

Thomas et al. reported that the most critical factor for successful replantation is the maintenance of viability of periodontal ligament cells.[20] Replanted tooth can undergo either inflammatory or replacement resorption[1] of which former's development depends upon both the degree of damage to the periodontium at the time of avulsion and the extent to which the viability of cells of periodontal ligament cells is maintained, whereas latter develops either after the damage of the periodontium at the time of accident and the presence of bacteria within the root canals and dentinal tubules.[21,22,23] Hence, the success of avulsed tooth replantation is dependent of viability periodontal ligament cells. Therefore, the predominant philosophy derived from the research of **Andreasen** and **Hjorting- Hansen,** for the treatment of avulsed teeth, is: **Replant the tooth immediately or as quickly as possible after the avulsion.**[1,24]

Immediate replantation of the avulsed tooth is the treatment of choice to re-establish the natural nutrient supply of the periodontal ligament cells and enhance the healing process.[20] **According to Andreasen** and **Hjorting-Hansen,**[1,24] teeth that are replanted quickly (within 30 minutes) have a better success rate than those that had longer extra oral time. **Andreasen et al.**[1]**, Soder PO et al.**[25] **Lindskog S et al.**[26] and **Blomlof L**[27] had indicated that storage medium is a more critical prognostic factor than the extra-alveolar period. So, physiologic storage media such as milk, saliva, saline, HBBS and Viaspan have been used for preserving the viability of periodontal ligament cells.[28,29]

HBSS, being expensive and not easily available at the site of accident, hence cost effective and easily available alternative materials like milk, saline, egg white, ORS and aloe vera have been found to maintain the viability of PDL.

In the present study eight different storage media were selected: HBSS (Group 1), Milk (Group 2), Saline (Group 3), Propolis (Group 4), Egg white (Group 5), Aloevera

(Group 6), Artifical saliva (Group 7), Oral Rehydration solution (Group 8)

Cell viability score in different storage medium at 45 minutes (Table 1, 2, 3 & Graph I)

HBSS showed the highest number of viable cells after 45 minutes followed by propolis, egg white, aloevera, milk, saline**,** ORS and artificial saliva. **Krasner and Person**[30] observed similar results and stated that HBSS is effective in preserving periodontal ligament cells of avulsed teeth, renews the degenerated periodontal cells and maintained a superior success rate if an avulsed tooth is soaked in it for 30 minutes.

Milk as a storage media showed more viable cells in comparison to saliva and the obtained results were in correlation with **Lindskog et al.**[31] who compared saliva and milk groups and concluded that saliva was less suitable than milk because of low osmolality and higher risk of bacterial contamination.

Egg white maintained more numbers of viable cells as compared to Aloe vera and these results were in correlation with **Badakhsh S et al.**[32] who compared egg white and 100% aloe vera and found that egg white maintained more number of viable cells because it has high nutrients properties and desirable pH and osmolality for being effective in preserving cells viability. He reported that pure aloevera is highly viscous at the time of experiment, which entirely covered the surface of the experimented cells and may have possibly prevented the accessibility of oxygen to them. The mean pH level of the 100% concentration of aloe vera is a bit acidic (mean pH level = 5.21) which results in decreased oxygenation & ultimately cell death.

Artificial saliva showed the lowest number of viable cells after 45 minutes and these results were in correlation with **de Sousa et al.**[33] who compared milk, egg white and artificial saliva after 1 hour of extraoral time and reported that the teeth stored in milk and egg white found more collagen fibre organization and cell numbers but artificial saliva had an inferior result because of disorganization of collagen fibers. **Malhotra N et al.**[34] also reported that saliva can be considered to be an acceptable short term-storage medium (less than 30 minutes) and its use should be limited to cases where the extra-alveolar duration is less and other superior storage media are not available.

Cell viability score among different storage media at 2 hours (Table 4,5,6 & Graph II)

Propolis showed highest number of viable cells after 2 hours followed by HBSS, and rest other media's these results were in correlation with **Martin and Pileggi**[17] and **Ozan et al.**[35] who compared propolis, milk, saline and HBSS, and observed that propolis was significantly more effective than HBSS and milk at 3, 6, 24, and 72 hours. They also found that milk kept significantly less number of viable PDL cells as compared to HBSS, as found in the present study.

Gjerston et al.[36] reported that propolis decreased the apoptotic levels of PDL fibroblasts and increased mitochondrial enzymatic activity of PDL cells when compared with HBSS. Hence, propolis can be a more beneficial storage medium for avulsed teeth as compared with HBSS.

Milk maintained more number of viable cells as compared to saliva in the present study and results were in correlation by **Courts et al.**[37] who found that milk is a significantly a better preservative of PDL fibroblast viability than saliva, water or air drying, but not as good as HBSS. Milk because of its easy availability & various properties like neutral pH, isotonicity, minimal bacterial content due to pasteurization, presence of protective enzyme and epithelial growth factors which stimulate regeneration and proliferation with high availability has been recommended by **International Association of**

Dental Traumatology[38] and the **American Academy of Pediatric Dentistry.**

Cell viability score among different storage media at 6 hours (Table 7,8,9 & Graph III)

Propolis showed highest number of viable cells even after 6 hours followed by HBSS, and rest other media's and these results were in correlation with **Mori GG et al.**[39] who found propolis as a better storage media for avulsed teeth and a 6 hours period of storage was more appropriate than 60 minute of storage as explained by **Buttke and Trope**[40] who states that storing avulsed teeth in medium that contain antioxidants might increase replantation success.

One of the major components of propolis is flavonoids, the most important pharmacologically active constituent and powerful antioxidant, which would explain its ability to maintain cell viability. Propolis also has an antibacterial property, that assists with successful replantation and decreases the chance of inflammatory resorption, a common sequelae in delayed replantation.[41]

Khademi et al.[42] compared milk and egg white as solutions for storing avulsed teeth, and the results have shown that teeth stored in egg white for 6-10 hours had a better prognosis as compared to those stored in milk for same amount of time .The findings of this study are in correlation with the results obtained in milk and egg white groups.

He also reported that there was no significant difference between egg white and HBSS, and also found egg white to be superior to tap water and milk. Egg white can be considered as a better & economically storage media due to its high nutrient value & easy availability.

Cell viability score among different storage media at 24 hours (Table 10, 11, 12 & Graph IV)

Propolis showed significantly highest number of viable cells after 24 hours than HBSS, egg white, aloevera, milk, saline, ORS and artificial saliva and the results were in correlation with **Martin and Pileggi**[17] and **Ozan et al.** [35]who stated that teeth stored in propolis demonstrated the highest viability of PDL cells, when compared to milk, saline and HBSS. They found that propolis was significantly more effective than HBSS and milk at 24 hours. **Krell**[43] also reported that viability of PDL fibroblast is maintained as long as 20 hours in propolis.

In our study saline, ORS and artificial saliva showed least number of viable cells and these results were in accordance with **McDonald RE et al.**[44] where they reported that water, saliva and saline were ineffective on maintaining PDL cell vitality because of bacterial contamination or the hypotonic effect that lead to the death of PDL cells.

Lindskog et al.[26] suggested that the low osmolality of saline in combination with bacteria which adhere to the PDL made it less desirable as a long term storage media. In the present study, as the time advanced, number of viable cells decreased in saline.

The osmolality of ORS is comparable but the pH is significantly less than the ideal storage media which may be the reason for comparatively lesser number of viable cells as found in ORS group in our study. The number of PDL cells at 24 hours storage time of propolis is comparable with the number of viable cells at 45 minutes in ORS and artificial saliva groups. This supports propolis as a long term storage media because of its antibacterial, antimicrobial, anti-inflammatory, antioxidant actions and healing occurs when the tooth is kept in this medium for a higher time. There was a steady decline in number of viable cells in all experimental storage media as time passed which was depicted in **Graph (V).**

The results of the present study revealed the effectiveness of the propolis keeping more viable cells even at 24 hours, However further research is essential to determine its

capacity in reducing or preventing the sequelae which frequently occur following replantation; i.e root resorption.In our study, teeth were directly stored in different storage media for checking the efficacy and were assessed for number of viable periodontal ligament cells at different time intervals. So, it could not simulate the natural conditions at the time of avulsion injury where the tooth directly contacts the open environment which leads to dehydration of cells prior to their storage in a suitable media.

Conclusion

1. Propolis showed the best results via maintaining the maximum viable cells at 2,6 and 24 hours followed by HBSS, Egg white, Milk, Aloe vera, Saline, ORS and Artificial saliva.
2. Hank's balanced salt solution was superior amongst all only at 45 minutes storage time intervals.
3. Artificial Saliva was the least effective storage and milk can also be considered as a storage medium provided the time duration not more than 2 hours as further on its properties declines.
4. Egg white showed comparable good results similar to Propolis and HBSS and can be considered as good storage media alternative due to easy availability and cost effectiveness.

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Legends Figure

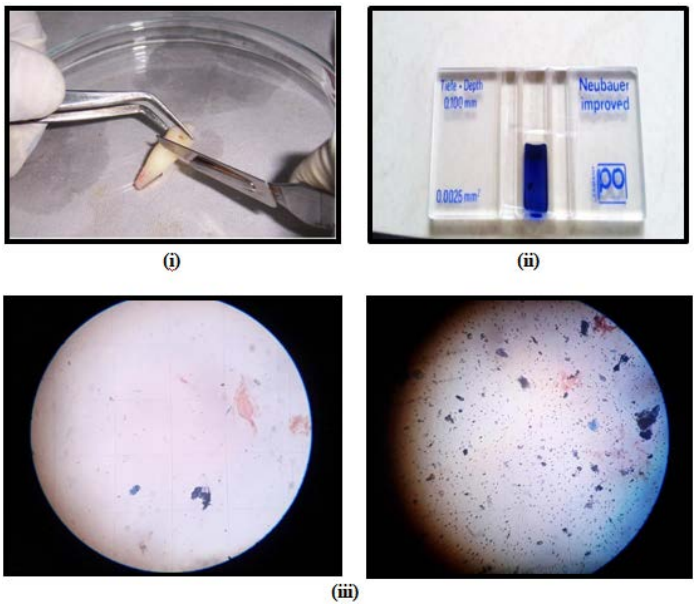

Fig 1:Various steps in the procedure

(i) PDL Cells were scraped from the apical 2/3rd of the root

(ii) Staining of the cells with 0.4% Trypan blue stain

(iii) Microscopic view of viable and nonviable cells