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Comparative Evaluation of Ice Apple Water and Egg White with HBSS as a Storage Medium to Preserve Viability of Human Periodontal Ligament Fibroblasts: An in-Vitro Study

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Abstract: ***Aim: To evaluate the efficacy of Ice apple water (IAW) and Egg white in preserving the PDL cell viability in comparison to Hanks balanced salt solution (HBSS) Methodology: 24 premolars extracted for orthodontic therapeutic purposes were randomly and equally divided into three groups, Group I (Ice Apple Water), Group II (Egg white), Group III(HBSS) 8 teeth in each group. After the teeth were extracted, the teeth were kept in their respective storage media for 60 minutes, PDL from middle & apical region were scraped using No.15 Blade and subsequently incubated in Falcon tube for 30 minutes in a mixture containing 2.5 mL of phosphate buffer and 0.5 mg of type I collagenase and falcon tubes were centrifuged for five minutes at 800 rpm. The cells were labeled with 0.4% trypan blue for determination of viability. Trypan Blue stains non-viable cells blue and viable cells appear colourless or pink. The number of viable PDL cells are counted under light microscope with hemocytometer at 40× magnification. Viabilty =100x [live cells/(live+dead cells)]. All the readings were noted and entered in the excel sheet. All data collected will be statistically analysed. Results: The assessment of cell viability with formula analysed using ANOVA test with p value <0.05 significant. The mean value of PDL cell viability for ice apple water was seen to be, 72.97±4.34 where as that of egg white was 74.80±2.89 while PDL cell viability in case of HBSS was the maximum with the mean value of 81.65±3.20 with p value 0.000181. The comparison of PDL cell viability was done between the 3 groups using post-hoc test with p value <0.05 as significant. PDL cell viability in case of HBSS was significantly more than that of egg white with mean difference, p value 0.0024659 PDL cell viability in case of HBSS was significantly more than that of ice apple water with mean difference, p value 0.0002145. PDL cell viability in case of ice apple water was similar to egg white with mean difference being, p value 0.5632173 Conclusion: Ice apple water and egg white because of good viability of cells, easier availability, and cost effectiveness can be used as a storage medium for avulsed tooth.***

Keywords: Ice apple water, egg white, periodontal ligament cells, storage medium, cell viability

1. Introduction

Tooth avulsion is the total displacement of the tooth out of its alveolar socket due to traumatic dental injury.[1] It is characterized by injury to the periodontal tissue and disruption of the neurovascular supply of the affected tooth.[2] The treatment of choice in such cases is to replant the tooth back into the socket. Preserving the PDL cells viability is the main objective in treating these lesions in order to avoid the difficulties brought on by postponed re-plantation. According to Andreasen et al., delayed re plantation is when re-plantation is carried out beyond 5 minutes of avulsion.[3] Consequently, immediate re-plantation of an avulsed tooth is the method that is most frequently recommended for its management. Unfortunately, the majority of these situations result in a delayed replantation of an avulsed tooth, which has a poor long-term prognosis. The most prevalent complication after re-plantation is ankylosis, followed by external root resorption.[4] When immediate re-plantation is not feasible, the avulsed tooth must be placed in an appropriate storage medium in order to maintain any remaining vitality before it can be replanted.

The ideal storage medium should be easily accessible, should be able to preserve cell viability, and must have essential nutrients, physiological osmolality, antioxidant properties, a neutral pH, adherence, and clonogenic capacity. A number of different synthetic and natural products have been studied for maintaining PDL cell viability.[5-7] So far, the most studied and approved storage media have been Hank balanced salt solution (Gibco, Waltham, Massachusetts, United States of America), which has favourable biological properties, and milk, which is easily accessible.[8]

Ice apple water, has been studied for its potential use as a storage medium for various applications. It contains a range of nutrients and antioxidants, has been found to have beneficial effects on cell viability and function. Additionally, the use of ice apple water as a storage medium could have environmental and economic benefits, as it is a natural and renewable resource that is widely available in coastal parts of India. Egg white is a good storage medium for teeth because it has a pH level that is similar to normal salivary pH and contains proteins that can help keep the PDL cells viable and it is also an ingredient readily available at home. Current study is aimed at evaluating the efficiency of Ice apple water and egg white as storage media in comparison to HBSS.

2. Materials and Methodology

Inclusion criteria:

1) Freshly extracted non-carious teeth for Orthodontic purpose with intact periodontium, crown and closed apices.

Exclusion criteria:

1) Teeth extracted due to Caries.
2) Teeth extracted due to a periodontal pathology.
3) Teeth that have undergone traumatic extraction.
4) Teeth without intact crown.

This in-vitro study was conducted on 24 freshly atraumatically extracted non-carious teeth for orthodontic purpose with normal periodontium, intact crown and PDL and closed apices that were collected (Figure1) and were randomly categorized into Group I(IAW), Group II (Egg white), Group III(HBSS) with 8 teeth in each group. Ice apple water is extracted using 18 Gauge needle with sterile syringe that has a filter. The teeth were kept in their respective storage media for 60 minutes (Figure 2). And then held with forceps from the coronal region, and the coronal 3 mm of PDL was scraped with a curette to remove cells that might have been damaged and then PDL from middle & apical region were scraped using No.15 Blade (Figure 3). The scrapped cells were subsequently incubated in Falcon tube for 30 minutes in a mixture containing 2.5 mL of phosphate buffer and 0.5 mg of type I collagenase (figure 4). Following incubation, the Falcon tubes were centrifuged for five minutes at 800 rpm (figure 5). The supernatant was discarded and the centrifuged residue was collected. The cells were labeled with 0.4% trypan blue for determination of viability. Trypan Blue stains non-viable cells blue and viable cells appear colorless or pink. The number of viable PDL cells was counted under light microscope with hemocytometer at 40× magnification (figure 6). All the readings will be noted and entered in the excel sheet. All data collected will be statistically analysed.

Determination of the number of cells (total and viable):
The cells were viewed under an optical microscope at 10X magnification. The number of cells (total and non-viable) was determined by counting the cells overlying a 4×1 mm^2 area of the Neubauer's chamber (Rohem India). Viable and non-viable cells were counted separately

Figure 1: Extracted tooth collection

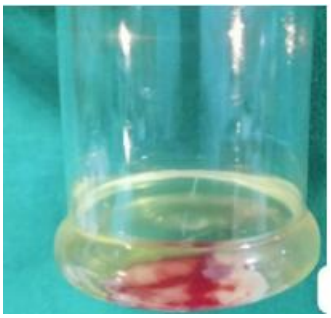

Figure 2: Storage of tooth in the allotted storage media

Figure 3: Scraping of PDL cells from middle and apical third

Figure 4: Adding cells to falcon tube for incubation

Figure 5: Centrifugation of the incubated cells

Figure 6: Counting the cells under light microscope

3. Results

The dead and live cell counts were tabulated and the data was analysed using SPSS software using ANOVA test with $p<0.05$ significant. The mean value of PDL cell viability for ice apple water was seen to be, 72.97±4.34) where as that of egg white was 74.80±2.89 while PDL cell viability in case of HBSS was the maximum with the mean value of 81.65±3.20 with p value 0.000181(Table 1).

Table 1: Comparison of Cell viability of PDL cells between three different Storage media assessed using ANOVA test

Parameter	HBSS	Egg white	Ice Apple water	P- Value
Cell viability (In terms of mean and standard deviation)	81.65±3.20	74.80±2.89	72.97±4.34	0.000181

PDL cell viability in case of HBSS was significantly more than that of egg white with mean difference, p value 0.0024659 PDL cell viability in case of HBSS was significantly more than that of ice apple water with mean difference, p value 0.0002145. PDL cell viability in case of ice apple water was similar to egg white with mean difference being, p value 0.56321(Table 2 and figure 7)

Table 2: Comparison of Cell viability of PDL cells between any two of the three different Storage media assessed using using post-hoc test

Parameter	Groups	Mean Difference (lower, upper)	p- value
Cell viability	Egg white solution – HBSS Salt Solution	-6.84 (-11.30, -2.38)	0.0024659
	Ice Apple Solution – HBSS Salt Solution	-8.67 (-13.13, -4.21)	0.0002145
	Ice Apple Solution – Egg White Solution	-1.83 (-6.29, 2.62)	0.5632173

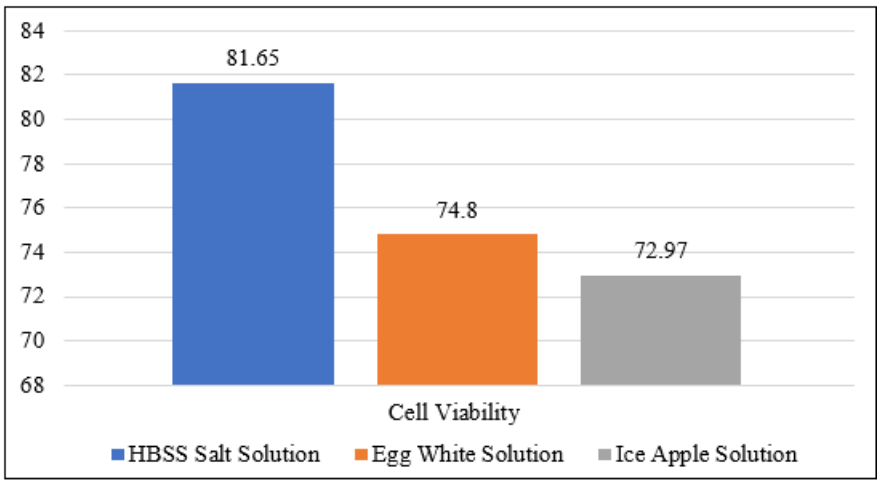

Figure 7: Comparison of Mean Cell viability of PDL cells between three different Storage media

4. Discussion

The reported incidence of tooth avulsion is approximately 16% of all traumatic injuries to the permanent dentition [8,9,10] The prognosis of the tooth depends on the measures taken at the time of the accident and/or the period immediately after avulsion. Although immediate replantation is the treatment of choice[9] , clinical experience has shown that most avulsed teeth are replanted only after an extended extra-alveolar time and when dry or stored in inadequately moist conditions. [8,9,10] The prognosis of replantation rests on the presence of viable cells in the PDL and on the proliferation to the injured zones of the root. This can be accomplished by instant replantation or through the storage of the tooth in a suitable medium for future replantation. When immediate replantation is not feasible, the tooth should be preserved in a medium that maintains PDL cell viability until conclusive treatment can be consummated.

Depending on the extra-alveolar time and the storage/ transport medium, pulp necrosis and degeneration of the cemental periodontal ligament (PDL) cells may occur. This may lead to inflammatory root resorption, which is the major cause of tooth loss. [8,11,12,13] Successful tooth replantation with a favourable prognosis depends essentially on the maintenance of PDL.[12,13,14] Several interim transport media for avulsed teeth have been investigated with respect to their capacity to preserve the vitality of PDL components and dental pulp tissue until the moment of replantation. [12,14]

There are various storage media available that can maintain the viability of PDL cells. The ideal storage media for an avulsed tooth must have following characteristics: Neutral pH, physiological osmolarity, antimicrobial properties and easy availability.
In 1994, the American Association of Endodontics guidelines for the Treatment of the Avulsed Permanent Tooth recommended Hank's Balanced Salt Solution (HBSS) as the appropriate transport medium for the avulsed tooth which could not be replanted instantly. However, the main disadvantages of HBSS are high cost and lack of availability.

Various other materials have been evaluated for the same purpose. The traditional storage media evaluated were not able to maintain the initial level of cell viability throughout the long-term period. So the present study aimed to evaluate

PDL cell viability in Ice apple water and egg white in comparison to HBSS.

Egg white was chosen to be a good storage medium for an avulsed tooth primarily because it contains a variety of proteins and nutrients that help support cell survival and metabolism, has a favourable pH and osmolality that supports cellular viability, abundant water content that could prevent dehydration of PDL cells, being relatively sterile reducing the risk of microbial contamination during storage and most importantly readily available and accessible at a low price.

Ice apple is a local fruit available in the coastal regions of India during sum mertime. It is the juicy, fleshy fruit of the sugar palm tree known to be packed with rehydrating properties and a cooling effect. Ice apple water would also make a good storage medium because it is rich in water, minerals (calcium, potassium, iron, phosphorus, zinc, and nutrients (vitamins A, C) closely mimicking the internal environment of human cells, and phytochemicals with antioxidant and anti-inflammatory properties. These helps protect and nourish PDL cells reducing oxidative stress and cellular damage during storage. Osmolality and pH are close to the physiological levels and reduced chance of contamination, while it is also readily available in the costal parts of India.

The use of extracted teeth to simulate avulsion has been recommended in previous studies.[19] Function of fibroblast is known to be affected by age, trauma, and inflammation. Therefore, mature, non-carious human premolars were selected for this study from young, healthy individuals without periodontal disease extracted for orthodontic therapeutic purposes. Pohl Y, Filippi A, and Kirschner H (2005)[20] stated that 15 minutes of dry time is the time when PDL cells remain in the non- compromised state. Following the 15 minutes dry time, the teeth were placed in different storage media for 60 minutes after rinsing in normal tap water. Panzarini et al. (2008)[21] also stated that 60 minutes of extraoral dry time is considered to be critical. The critical period where reduced cell viability was observed was at 60 minutes, and due to this reason in the present study, the tooth was stored in storage media for 60 minutes.

Then in the present study, the obtained scrapings were subsequently added to a Falcon tube (Ambion) containing 2.5 ml of Phosphate buffer (Gibco, Thermofisher). PBS (phosphate-buffered saline) is a balanced salt solution and can be used as cell culture applications like washing cells before dissociation, transporting cells or tissue, diluting cells for counting, and preparing reagents. To quantitate the number of viable PDL cells in the current study and to preserve maximum cell viability, the root surfaces were treated with 0.5 mg of Type I-Collagenase enzyme (Gibco, Thermofisher) for rapid cell retrieval and cellular integrity. In the current study, The Trypan blue dye exclusion technique for determination of cell viability was used as it is quick and simple to perform.[22] The reactivity of the stain is based on the observation that the chromophore present on the cell membrane is negatively charged, because of which it fails to take up the stain unless there is damage to the membrane.[23] Thus, viable cells are visible as clear cells as they do not take up the stain while nonviable cells take up the stain and appear violet-indigo colored.

To evaluate the efficacy of the different storage media in preserving the viability of dental fibroblasts, Ragnarsson's [24], and Doyle's[25] methods are extensively quoted in the various literature. In Ragnarsson's method, the fibroblasts are first removed from the root surfaces and added to the storage media for culturing.[24] The viability of cells was evaluated at different time intervals and counted. In Doyle's method, the extracted tooth is directly placed in the storage medium.[25] After a predetermined time, the tooth is removed from the medium, and PDL cells are isolated to evaluate cell viability. In the present study, Doyle's method was followed because it more closely replicates the actual clinical scenario.

The result of the present study demonstrates that significantly more viable cells survived in HBSS (81.65±3.20) among all groups because HBSS is a culture medium with an outstanding capacity for maintaining the vitality of cells of periodontal ligament and that the cells stored in HBSS did not show any distortion of morphology.[25] In inter-group comparison, HBSS showed significant results from all other experimental media. Egg white maintained an average number of viable cells (74.80±2.89%) in the present study which can be attributed to its high albumin content that provides essential proteins and buffers pH fluctuations, enhancing short-term cell survival[26,27], but the viable cell count is fewer than HBSS which is possibly accredited to the high pH (9.38) and also to a higher amount of proteins in egg white that may potentially act as a foreign body. Khademi A et al.[28] showed that there was no significant difference in PDL cell viability between the egg white and HBSS group, and they were superior to tap water and milk.[28]

IAW demonstrated significant potential (72.97±4.34) in maintaining PDL cell viability. This aligns with prior research indicating IAW's osmolarity and nutrient composition (e.g., sugars, electrolytes) create a favorable environment for cellular integrity 29,30. Bijlani et al. reported IAW's effectiveness over 24 hours, attributing its success to biocompatible phytochemicals that reduce oxidative stress29 Its high water content (94–96%) and natural sugars (glucose/fructose) mimic extracellular fluid osmolarity, preventing cell rupture 29,30. Antioxidants (e.g., phenolics) may further mitigate ischemia-induced damage30. Similarly, IAW's performance in our study underscores its viability as an economical, natural alternative. Cell viability in ice apple water is similar to that of Egg white, the difference between the two is insignificant and both have viability more than 70% which means they are both promising in maintaining PDL cell viability.

5. Limitations and Future Directions

- In vitro constraints: 2D cell cultures lack the biomechanical complexity of intact PDL tissue. Future studies should incorporate 3D models or ex vivo tooth models.
- Temperature variability: Most evaluations (including this study) used room-temperature storage. Testing efficacy across temperatures (4–37°C) would better reflect real-world conditions .

- Component optimization: Isolating active compounds in IAW (e.g., via HPLC) could enhance standardized formulations.

6. Conclusion

All media are capable of being used as transport storage media as they showed cell viability of more than 70%. Within 60 minutes, and at room temperature, Hank's balanced salt solution (HBSS) is the most effective storage medium. Ice apple water and Egg white can be good alternatives to HBSS because they are readily available and inexpensive. Due to the superior viability, easier availability, and cost- effectiveness, Egg white, and Ice apple water can be recommended as a viable storage medium and alternatives to HBSS.

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