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# **Comparative evaluation of periodontal ligament cell viability of permanent teeth in five different storage media followed by simulated avulsion injury: An in vitro study**

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**Abstract---**Aim: To evaluate and compare the viability of periodontal ligament cells of avulsed teeth in five different storage media followed by simulated avulsion injury. Settings and Design: Seventy-five premolars extracted for orthodontic therapeutic purposes were randomly and equally divided into five groups based on storage media used [A: HBSS (control), B: Milk (experimental), C: Aloe Vera (experimental), D: Egg white (experimental), E: Coconut water (experimental)]. Methods and Material: Following extractions, the teeth were placed in one of the five different storage media for 60 minutes, following which the scrapings of the PDL were collected in Falcon tubes which already contained collagenase enzyme in 2.5 mL of Phosphate buffered saline. The tubes were subsequently incubated and centrifuged. Then acquired PDL cells were stained with Trypan Blue dye and were counted under an optical microscope. Statistical Analysis Used: Results were subjected to statistical analysis using ANOVA test and Post Hoc Tukey test. *p-value* < 0.05 is considered to be significant. Results: HBSS

showed the highest percentage of viable cells (80.14%), followed by Egg white (74.01%) and Aloe Vera (73.68%). Milk and Coconut water showed the least percent of viable cells 63.20%, 63.58% respectively. There is no significant difference in PDL cell viability between Aloe Vera and Egg white ( $p$  value = 0.999), but both the groups showed higher and significant results than milk and coconut water group. Conclusion: Due to the superior viability, easier availability, and cost-effectiveness, Egg white, and Aloe Vera can be recommended as a viable storage medium and the best alternative to HBSS. Clinical Significance: Despite the existence of better quality storage media such as HBSS, the lack of availability of these products at the place and moment of the accident makes their recommendation questionable. On the contrary, Aloe Vera and egg white are a commonly available media which is inexpensive also. Hence the chances of availability of Aloe Vera near the site of injury as well as school premises could be relatively high.

**Keywords**---Tooth Avulsion, Avulsion Storage Media, Periodontal Ligament Fibers, Reimplantation, Cell Viability, HBSS, Aloe Vera, Milk, Egg White, Coconut water.

## Introduction

The oral and maxillofacial region trauma occurs very frequently which comprehends 5% of all injuries for which people seek treatment.<sup>[1]</sup> Dental injuries are the most prevalent facial injuries of which crown fractures and luxation occur most often. Traumatic injuries to permanent anterior teeth are common during childhood, and 0.5–16% of the 7 to 10 year age group experience tooth avulsion.<sup>[2]</sup> In permanent dentition, many of these teeth are knocked-out during daily activities or sporting events as the result of fights or sports injuries, while falls against hard objects. Sports account for 60% of Traumatic Dental Injuries (TDI), and schools are the place where one can find a noticeable risk of TDI. <sup>[3]</sup> An Exarticulation is defined as the complete displacement of a tooth from its alveolar socket due to traumatic injury by World Health Organization (WHO).<sup>[4]</sup> This form of injury is important because it most commonly involves maxillary central incisors, which makes a major esthetic contribution to the smile.

Absolute treatment for an avulsed tooth is its immediate replantation into the socket, which significantly improves the prognosis. Andreasen reported in his retrospective study that 90% of avulsed teeth could be successfully retained when they were replanted within the first thirty minutes of the accident. <sup>[5]</sup> When immediate replantation of an exarticulated tooth is not possible, the PDLF (Periodontal Ligament Fibers) should be incubated in a physiological storage medium to maintain its viability during transportation to the dental office because dry storage of avulsed teeth leads to the death of PDL cells of the root. Partial or total loss of PDL leads to ankylosis since the activity of cells derived from the PDL plays a crucial role in the prevention of ankylosis. Both the storage media and extra-alveolar duration are significant critical factors in determining the final prognosis.

Until now, various types of wet storage media for avulsed teeth have been investigated, which may vary from cell and tissue culture solutions like Hank's balanced salt solution (HBSS); medical/hospital products developed specifically for organ storage purposes, like ViaSpan, Euro-Collins culture media, Minimum Essential Medium (MEM), Saline. However, the major disadvantage of HBSS and many of the other aforementioned media is that they are not easily available at places where these traumatic injuries occur. Other natural products like water, saliva, Bovine milk and its variations, Propolis, Green tea, Morus rubra (red mulberry), Egg white, and Coconut Water can be used as a storage media which are easily available.

An ideal storage medium should be capable of maintaining PDL and pulp cell viability while presenting clonogenic capacity, compatible physiological pH and osmolality, antioxidant property, no or minimal microbial contamination, maintained at an appropriate temperature, high availability, and low cost. [6] It also should be readily accessible, especially to families with children. Thus the purpose of this in vitro study is to compare the efficacy of five storage media, i.e. Milk, Aloe Vera, Coconut water, Egg white, and HBSS, in preserving the viability of PDL cells of avulsed teeth.

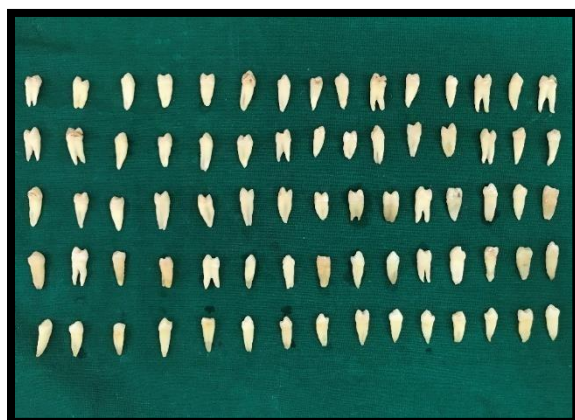
## Materials and Methods

Seventy-five human premolar teeth extracted for orthodontic therapeutic purposes were included in this study. Protocol approval was obtained from an Ethical Committee of Ahmedabad Dental College & Hospital. Around 75 sound extracted premolars were collected for the study from the Out-patient Department of Oral and Maxillofacial Surgery, Ahmedabad Dental College and Hospital, and from the Private dental clinic of Ahmedabad city.

Caries free maxillary or mandibular premolar with normal periodontium and closed apices, extracted for the orthodontic reasons were included. Teeth having PDL injury while extraction and teeth having the periapical disease were excluded.

### 5 Experimental groups with a sample size of 15 teeth in each group:

1. Group A: Hank's balanced salt solution was used.
2. Group B: Amul Taaza Pasteurized homogenous milk which contains 3 gm/ml fat was used at room temperature.
3. Group C: Aloe Vera leaves were collected. After the removal of the outer skin, the inner gel was collected and blended. It was filtered through a piece of muslin cloth and was collected into a glass container for use. For each sample, fresh leaves were used.
4. Group D: Egg white was separated from the yolk and collected in a glass container for use. For each sample, a new egg was used.
5. Group E: Fresh coconut water was used for each sample.



**Figure 1: 75 Extracted sound premolars**



**Figure 2: Storage media used for study**



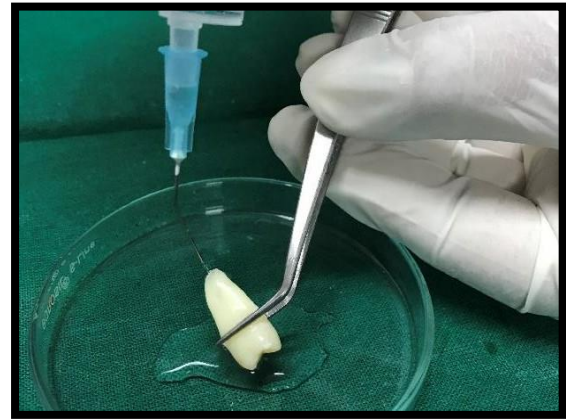
**Figure 3: Extraction of sound premolar teeth**



**Figure 4: Kept in mud for 10 minutes**



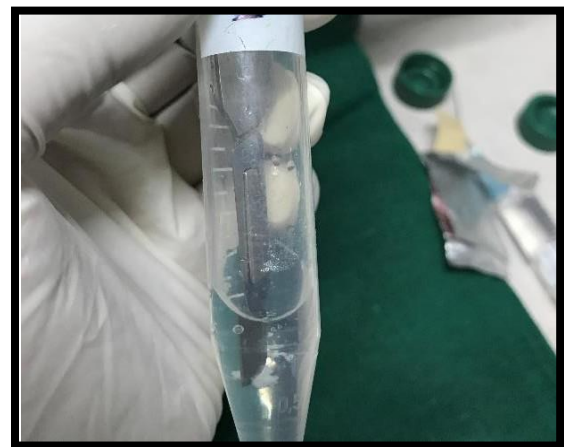
**Figure 5: Tooth in storage media for 60 minutes**



**Figure 6: Rinsing with normal saline**



**Figure 7: Scrapping of epithelium from apical-third of root**



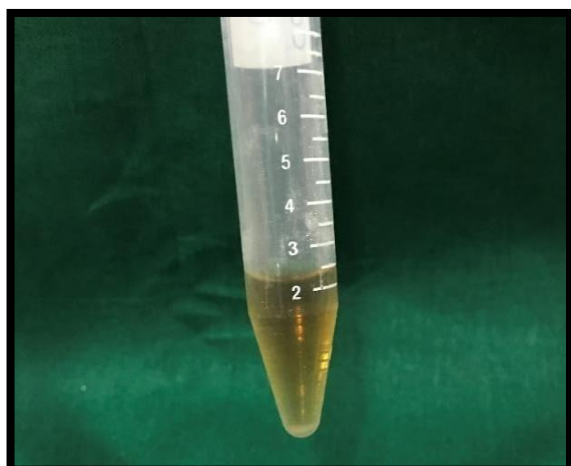
**Figure 8: Scrapped epithelium added to Falcon tube**



**Figure 9: Collagenase type – I added to Falcon tube**



**Figure 10: Falcon tube in incubator for 30 minutes**



**Figure 11: Centrifuged residue at bottom of Falcon tube**



**Figure 12: Stained Suspension added to Neubauer's chamber**

Caries free premolars extracted for the orthodontic purpose were collected for the study from the Out-patient Department of Oral and Maxillofacial Surgery, Ahmedabad Dental College and Hospital. Following extractions, the coronal third of PDL has been scraped with a curette (Jaypee, India) to remove cells likely to be damaged due to instrumentation while extraction. To simulate avulsion injury, teeth were kept in mud for 15 minutes following removal of the damaged cells from the coronal one third in a petri dish (Borosil). Before putting the tooth in respective media, the teeth were held with a tweezer by the crown and dipped twice in the water container to remove mud from its surface. The samples were placed in respective storage media for 60 minutes in a sterile plastic container (Necor).

After 60 minutes, the teeth were held with a tweezer by grasping the crown portion, and the root surface has been irrigated twice with sterile isotonic saline to remove the residual storage

media. Apical two-third of the root surfaces (as measured from epithelial attachment) were scraped with Bard-Parker blade No. 15(Surgeon) in a petri dish to obtain the periodontal ligament (PDL) cells. The obtained scrapings were subsequently added with a Micropipette of 100-1000  $\mu$ l (ErbaMannheim) to a Falcon tube (Ambion) containing 2.5 mL of Phosphate buffer (Gibco, Thermofisher). To this mixture, 0.5 mg of Type I Collagenase enzyme (Gibco, Thermofisher) was added, and the mixture was incubated in Incubator (Microlab Instruments, INCU-30) for 30 minutes. Following incubation, the Falcon tubes were centrifuged for five minutes at 1000 RPM. The supernatant was discarded with a Micropipette of 100-1000  $\mu$ l and, the centrifuged residue was collected. The cell suspension was prepared in HBSS. For that 0.5 ml, of 0.4% Trypan blue (Gibco, Thermofisher) solution was transferred to a test tube with Micropipette of 5-50  $\mu$ l. In the test tube, 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) were added and mixed thoroughly. Trypan Blue stains non-viable cells blue and viable cells appear color-less or pink.

The test tube was allowed to stand for 5 to 15 minutes. After 15 minutes with Micropipette of 5-50  $\mu$ l, a small amount of Trypan blue-cell suspension mixture was added to both chambers of the Neubauer's chamber (Rohem India) and covered with a coverslip. Then the cells were observed with an optical microscope (Labomed).

#### **Determination of the number of cells (total and viable):**

The cells were viewed under an optical microscope at 400 magnification (10 $\times$ ocular and 40 $\times$ objective). The number of cells (total and non-viable) was determined by counting the cells overlying a 4 $\times$ 1 mm<sup>2</sup> area of the Neubauer's chamber (Rohem India). Viable and non-viable cells were counted separately.

#### **The cells per ml was calculated as:**

Cells per ml = Average count per square  $\times$  dilution factor  $\times$  10<sup>4</sup> (count 10 squares)

The viable cell percentage was calculated as: Cell Viability (%) = [(Total cells - Stained cells)/Total Cells]  $\times$  100

#### **Statistical analysis**

All the obtained data were tabulated and evaluated using Statistical Package for the Social Sciences Software (SPSS version 20.0) for Windows. The number of viable cells from each storage media were analyzed using one way ANOVA test, and then each test group was compared with all other groups using the Post Hoc Tukey test. *p-value* < 0.05 is considered to be significant. Using the following tests, the statistical analysis was done

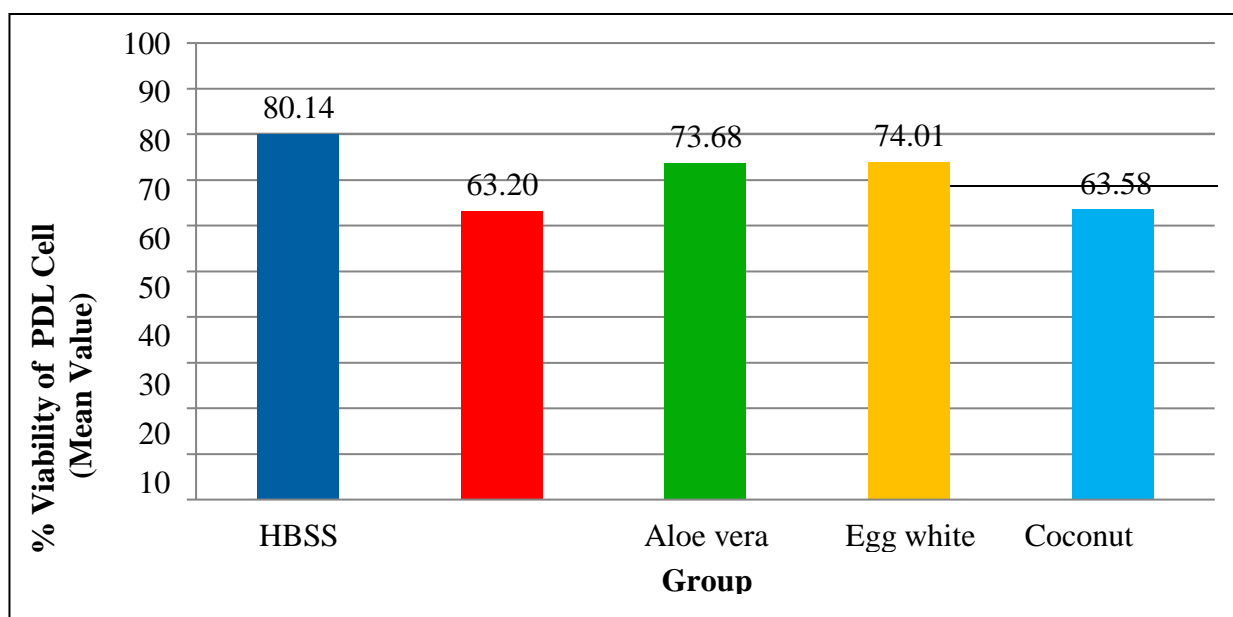
#### **Results**

The results obtained after checking the viability for all the 5 groups HBSS, Milk, Aloe Vera, Egg White and Coconut Water are shown in the following tables and charts. Table 1 and Chart 1 shows the mean and standard deviation of the viability of PDL cells in percentage for each group.

Table 1: Mean for PDL Cell Viability of all the group

Group	N	Mean (%)	SD (%)	Minimum Score (%)	Maximum Score (%)	<i>p</i> -value
Group A (HBSS)	15	80.14	4.47	74.01	87.83	<0.001 (S)
Group B (Milk)	15	63.20	3.29	56.63	68.89	
Group C (Aloe Vera)	15	73.68	2.82	68.88	77.94	
Group D (Egg White)	15	74.01	3.02	66.88	77.67	
Group E (Coconut Water)	15	63.58	4.91	66.30	54.29	

\*One way ANOVA, †SD: Standard Deviation, ‡ *p* - value: <0.05 (Significant), §S: Significant



**Chart I: Mean value (%) for PDL Cell Viability of all the groups**

Table 2: Intergroup comparison for PDL cell viability in different storage media

(I) GROUP	(J) GROUP	Mean Difference (I-J)	<i>p</i> -value
HBSS(A)	Milk(B)	16.94	<0.001
HBSS(A)	Egg white(D)	6.13	<0.001
HBSS(A)	Aloe vera(C)	6.46	<0.001
HBSS(A)	Coconut water(E)	16.56	<0.001
Milk(B)	Egg white(D)	-10.81	<0.001
Milk(B)	Aloe vera(C)	-10.47	<0.001
Milk(B)	Coconut water(E)	-0.38	.999 (S)
Egg white(D)	Aloe vera(C)	0.34	.999 (S)
Egg white(D)	Coconut water(E)	10.43	<0.001
Aloe vera(C)	Coconut water(E)	10.09	<0.001

\*Post Hoc Tukey test, †*p*-value: <0.05 (Significant), ‡S: Significant

Table 2 demonstrates intergroup comparison of different storage media. The teeth stored in HBSS demonstrated a significantly higher percentage of viable PDL cells (80.14%) compared to those stored in Milk (63.20%), Aloe Vera (73.68%), Egg white (74.01), and Coconut water (63.58%) ( $p$  value > 0.001). There is no significant difference in PDL cell viability between Aloe Vera and Egg white ( $p$  value = 0.999), but both the groups showed higher and significant results than milk and coconut water group.

## Discussion

To avoid root resorption and maintain PDL cell viability, various transport media have been proposed to store the tooth if immediate reimplantation is not possible. The basic principle behind the use of these media is to transport the tooth, maintaining an environment similar to that of the oral cavity. Various studies have found HBSS, ViaSpan, and eagle's medium to be suitable for the transportation of avulsed teeth as they preserved PDL cell viability. However, the main disadvantages of these media are high cost and lack of availability. Therefore an effort should be made to find a storage medium that is readily available and cost-effective.

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. In the present study, avulsion injury was simulated by extraction of the tooth and leaving it in mud for a minimum of 15 minutes. This is the time the victim and the attendant can consume to recover from the traumatic event and act appropriately. [7] Pohl Y, Filippi A, and Kirschner H (2005) [8] 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) [9] 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.

The fundamental principle for the storage of avulsed teeth is that they should be stored in a medium that most closely replicates the oral environment. [10] HBSS (Gibco, Thermofisher) was chosen as the positive control in the present study. HBSS is a standard saline solution, which is widely used in biomedical research to support the growth of many cell types. It is non-toxic, pH balanced, and contains many essential nutrients. A disadvantage of HBSS is that it may not be readily available at many locations in which tooth avulsions are likely to occur. [11] Milk was chosen as one of the experimental group in the present study because of its respective favorable characteristics as a storage medium for avulsed teeth; it is an isotonic liquid with an approximately neutral pH, contains growth factors, has low or no bacterial content, and essential nutrients for cells, in addition to having a high availability mostly everywhere and low cost. [6]

In the present study, Aloe Vera gel was chosen as a transport medium because it contains 99% water and over 75 nutrients, which include 20 minerals, 19 amino acids, and 12 vitamins. Many studies have illustrated that Aloe Vera has superlative properties, such as anti-inflammatory, antibacterial, antifungal, anticancer, and even antioxidant activities. [12, 13] The egg white was selected due to its nutritious constituents. The pH of egg white is 9.89. Egg white from a single egg contains 4.7 grams of 40 different proteins, 0.3 grams of carbohydrate, 62 milligrams of sodium, and the remainder being water. [14] It is considered a good choice as a storage media for teeth undergoing delayed replantation because apart from proteins, it also contains vitamins and water, the absence of microbial contamination, and easy access. [6, 15] In the present study, coconut water was chosen as a natural product that has high osmolality. Tender coconut water is biologically pure and it helps to replace fluids and electrolytes (potassium, sodium, and chloride). Taking these characteristics into consideration, we

hypothesized that this natural isotonic drink could be a viable storage media for the transportation of avulsed teeth.

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.<sup>[16]</sup> 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.<sup>[17]</sup> 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<sup>[18]</sup> and Doyle's<sup>[19]</sup> 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.<sup>[18]</sup> 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.<sup>[19]</sup> 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 (80.41±4.47) 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.<sup>[19]</sup> In inter-group comparison, HBSS showed significant results from all other experimental media. Aloe Vera maintained an average number of viable cells (73.68%) in the present study, which could be attributed to its composition. It contains a glycoprotein with cell proliferating activity.<sup>[20]</sup> Aloe Vera also contains allantoin, which has been found to stimulate fibroblast activity and collagen proliferation.<sup>[21]</sup> Aloe Vera and egg white showed the non-significant difference in PDL cell viability (0.34%).

In the present study, egg white (74.01%) maintained a fewer viable cells than HBSS (80.14%). This 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.*<sup>[22]</sup> 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.<sup>[22]</sup> Milk showed the least number of viable cells (63.20%), which could be attributed to the presence of various enzymes, which are harmful to the fibroblasts of the PDL, and there is also a lack of a cell energy source and ions which doesn't permit repopulation of PDL cells.<sup>[23]</sup> Gamsen *et al.* (1992)<sup>[24]</sup> found that milk was unable to regenerate depleted cell metabolites and restore the viability of PDL cells. Milk is found to be a compatible storage medium only when it is cold and fresh. Sigalas E, Regan JD, Kramer PR, Witherspoon DE, and Opperman LA suggested that milk could be used as a storage medium for PDL cells only when it is chilled.<sup>[11]</sup> There is no significant difference in the mean PDL cell viability between milk and coconut water (0.38%).

Coconut water in the present study showed poor cell viability (63.58%), which is highly significant with all other groups except milk. These results may be attributed due to its acidic pH (4.1), which is deleterious to cell metabolism. A study done by Moreira-Neto JJ *et al.* (2009)<sup>[25]</sup> showed that coconut water was less effective as a storage medium than coconut water with

sodium bicarbonate, which indicates that the pH is an important factor to be considered for the preservation of cell vitality. During the neutralization of the pH, the osmolality of the coconut water increased, which maintained the viability of the fibroblasts. Moura CC *et al.* (2014) [26] in the previous study stated that pH-adjusted (7.0) coconut water showed promising results as a storage solution for avulsed teeth up to 24 hours.

## Conclusion

All media are capable of being used as transport storage media as they showed cell viability of more than 60%. Within 60 minutes, and at room temperature, Hank's balanced salt solution (HBSS) is the most effective storage medium. Aloe Vera and Egg white can be a good alternative to HBSS because they are readily available and inexpensive. The viability of cells in Milk and Coconut water inferior to all groups. Due to the superior viability, easier availability, and cost-effectiveness, Egg white, and Aloe Vera can be recommended as a viable storage medium and best alternative to HBSS.

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