

# Assessment of Viability of Periodontal Ligament Cells in Different Storage Media

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## ABSTRACT

**Aim & Objectives:** To assess the viability of periodontal ligament (PDL) cells in different transport media, at different time intervals. To decipher an effective, economical and easily available transport medium. **Study Design:** 180 premolars undergoing orthodontic extraction were chosen for the study and segregated into 9 groups containing different storage media. After the stipulated time intervals, the viability the PDL cells was tested. **Results:** On statistical analysis at 5 different time intervals of 1, 2, 4, 6 and 24 hours, it was found that PDL cell viability varied in different media and at the different time intervals; with low fat milk having the highest PDL cell viability count and tap water having the least. **Conclusion:** Low fat milk is the most effective media in maintaining the viability of PDL cells upto 6 hours, after which its efficiency declined, whereas contact lens solution showed consistent results and maintained the highest number of viable cells upto 24 hours. Gatorade showed the least amount of cell viability.

**KEYWORDS:** Dental trauma, Exarticulation, Transport media, Periodontal cell viability

## INTRODUCTION

Children will be children all throughout the world. Their languages may differ, but their smiles speak volumes. They are always eager to discover the world around them, often challenging their powers without noticing the dangers they are exposed to.

Among dental injuries, exarticulation is one of the most intricate forms, characterized by the complete expulsion of the tooth from the alveolar socket. It constitutes around 1- 16% injuries to the permanent anterior teeth.<sup>1</sup>

The greatest success of a replanted exarticulated tooth occurs when it is replanted immediately, which may not always be possible.<sup>2</sup> Hence, the periodontal (PDL) cell viability becomes mandatory till the tooth is replanted.

Immense research to maintain the viability of periodontal ligament cells has been carried out while newer media are still under scrutiny.

A transport medium that is economical, readily available at the accident site, allowing the safe transport of the exarticulated tooth to a trained dentist for a successful reimplantation is required. A wide range of media has been proposed. There is still continuous research in this field. So, there is a necessity to come out with an effective, readily available and economically favourable storage media for the general public which is ideal to preserve the viability of PDL cells.

### Aim:

- To assess the viability of periodontal ligament cells in different transport media namely high, medium and low fat milk, coconut water, tap water, egg white, saline, gatorade and contact lens solution at 5 different time intervals of 1, 2, 4, 6 and 24 hours.

- To decipher an effective, economical and easily available transport medium.

## MATERIALS AND METHOD

This study was conducted in the Department of Pediatric Dentistry. 180 caries free human premolars with apparently normal periodontium and closed apices undergoing extraction for the purpose of orthodontic treatment were selected for the study. The extractions were performed atraumatically with utmost care. Following extraction, the teeth were held with forceps at the coronal region and the coronal 3mm of PDL on the root surface was scraped from the cervical margin using No.15 BP blade to remove the cells that might have been damaged during extraction. The teeth were then randomly divided into 9 groups of twenty teeth each and were transferred to each storage media namely low, medium



Fig 1. PDL Cells were scraped from the apical 2/3rd of the root

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and high fat milk, tender coconut water, egg white, tap water, saline, contact lens solution and gatorade which were stored in sterile test tubes. Every tooth was maintained for different time intervals i.e., for 1, 2, 4, 6 and 24 hours. All the procedures were implemented at room temperature. The groups were:

- Group 1: Low Fat Milk
- Group 2: Medium Fat Milk
- Group 3: High Fat Milk
- Group 4: Coconut water
- Group 5: Egg white and
- Group 6: Tap water
- Group 7: Saline
- Group 8: Gatorade and
- Group 9: Contact lens solution (Aqua Soft Contact Lens Solution)

**Harvesting of PDL cells:** After the stipulated time interval, the teeth were taken to the pathology laboratory, where further procedures were done. The teeth were handled by the anatomical crown during the procedures in order to prevent damage to the periodontal cells. All the teeth were then held by a tweezer by grasping the coronal portion and cleansed by irrigation with phosphate buffered saline {PBS} to remove the debris, storage media, etc. The teeth were incubated for 60 minutes in 10 ml test tubes with a 2.5 ml solution of 0.2 mg/ml-1 of Collagenase CLS 2 in PBS. After incubation, 50µl of fetal bovine serum was added to each tube. The tube was then centrifuged for 4 minutes at 1000rpm. The supernatant was removed with sterile micropipettes. The apical two third of the roots were scraped using number 15 BP blade to obtain periodontal tissue (Fig 1). These scrapings were transferred onto sterile slides, and the cells were then stained with 0.4% trypan blue for determination of the viability of the cells (Fig.2).

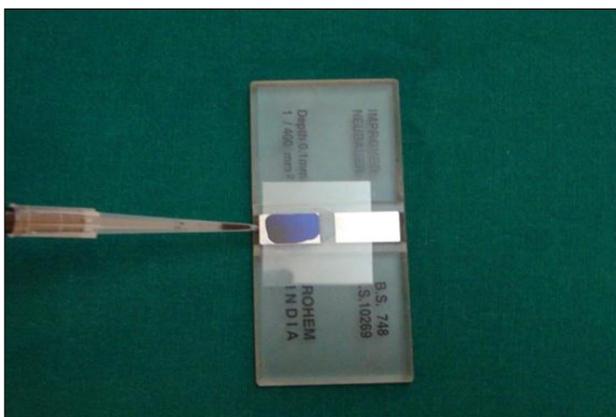


Fig 2. Staining of the cells with 0.4% Trypan Blue stain

The number of viable and nonviable cells were counted under a light microscope with a Haemocytometer at 20X magnification. The nonviable cells did not take up the stain (Fig.3) whereas the viable cells took up the stain (Fig.4).

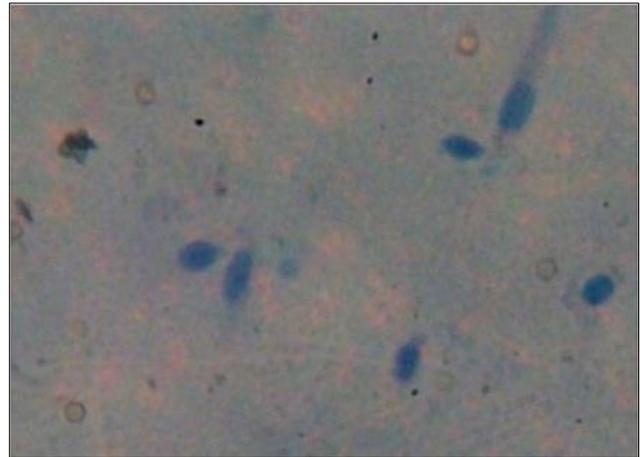


Fig 3. Non-viable cells took up the stain



Fig 4. Viable cells did not take up the stain

The viability percentage of the cell population of each sample was obtained by applying the mathematical equation;  $(UC/TC) \times 100 = \%$

Where UC was the unstained cell count (viable cells) and TC was the total cell count (stained + unstained cells)

There are two methods for evaluating the effectiveness of different storage media in preserving the viability of dental fibroblasts. The method most commonly used, is to scrape off fibroblasts from the root surfaces first and then add them to a storage medium for culturing. This is followed by the evaluation of viability of cells at different time intervals.

In another method, the extracted tooth is directly placed into the storage medium. After the stipulated time, the PDL cells are secluded using enzymes and simultaneously the tooth is taken out of the medium to assess cell viability. This method is similar to the primary cell culture. This particular methodology was used in this study.

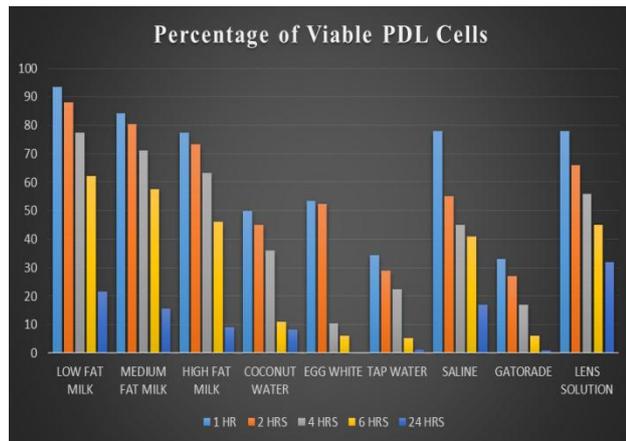
## RESULTS AND OBSERVATIONS

One-Way ANOVA and Tukey post hoc test were used for the statistical analysis of viable cell count under a

light microscope using a haemocytometer. The random and equal distribution of the 180 orthodontically extracted teeth was done within 9 study groups. (Table 1, Graph 1)

Group	1 hr	2 hrs	4hrs	6hrs	24hrs
1	93.57	87.98	77.52	62.27	21.67
2	84.11	80.32	71.19	57.52	15.60
3	77.47	73.39	63.25	46.07	9.23
4	49.99	45	36	10.9	8.26
5	52.34	52.43	10.36	6.07	0
6	34.40	29.10	22.33	5.4	1.18
7	78	55	45	41	17
8	33	27	17	6	1
9	78	66	56	45	32

Table 1. Mean % of viable PDL cells in study group at different time intervals



Graph 1. Percentages of viable PDL cells in different storage media

## DISCUSSION

Whenever we visit any hospital, the first thing we can notice is the 24\*7 care centre. In trauma, there is an acute and unexpected injury which needs immediate attention in order to prevent damage to the tissues. Same is the case concerning injuries to the oral structures. Dental avulsion is one such traumatic injury which is characterized by the total displacement of the tooth from its socket, which damages the PDL, cementum, dento-alveolar complex, gingival and the dento-pulpal tissues.<sup>1</sup> The most indicated procedure for this kind of dental trauma is immediate reimplantation, which is a surgical technique consisting of inserting the tooth back into its socket.<sup>3</sup> Trauma may occur at any place like school, playgrounds, on roads, etc. It is practically impossible to reimplant immediately. Hence, for a successful reimplantation, it is important to store the tooth in a proper, suitable storage media. These storage media should preserve the life of the cells of PDL during the time they are out of their alveolar socket. They help in better prognosis as they retain the vitality of the PDL and help in the proliferation of the PDL cells on the damaged areas of the root.<sup>4,5</sup> It is thus necessary to educate the common man, especially the parents and the school teachers regarding such media so that the tooth can be brought to the dental clinic under optimal conditions, which will help the dentist render better treatment. This

will also help the dentist in contributing a better quality of life for the patient.

The vitality of the PDL on the root surface enhances the probability of reinsertion of dental fibres with the alveolar ones.<sup>6</sup> The maintenance of the vitality of PDL cells attached to the tooth is lower in a dry environment. The tooth should necessarily remain in a humid place.<sup>3,6-13</sup> The use of these media is mandatory when immediate reimplantation cannot be done.<sup>14</sup> The lapse of time between the accident and the treatment also plays an important role, the greater the time lapse, the lesser is the prognosis. Nevertheless, the use of an inappropriate storage medium can increase the rate of cell necrosis and result in ankylosis or root resorption. The fundamental philosophy for the storage of exarticulated teeth is that the teeth should be stored in an environment that is closely replicates the oral environment. The normal metabolic, morphologic and physiologic conditions of the teeth should be attained as accurately as possible. Numerous studies have tried to determine the optimum storage media for PDL cell viability and preservation in different conditions.<sup>15-17</sup>

There are two methods for evaluating the effectiveness of different storage media in preserving the viability of dental fibroblasts. The most common method, (Ragnarsson's method 1985)<sup>18</sup> is to first remove fibroblasts from the root surfaces and add them to a storage medium for culturing. The PDL cell viability is evaluated at different stimulated time intervals and the cell line is used for the test in this method. In another method (Doyle et al. in 1998)<sup>19</sup> the extracted tooth is directly placed in the storage medium. After the pre-determined stipulated time, the cells are isolated with the help of enzymes and the tooth is removed from medium to evaluate the viability of the cells. This method is similar to the primary cell culture. Both methods have their own advantages and disadvantages. The principle plus point of the first method, is that a fewer number of teeth make a larger number of fibroblasts available. However, the biggest disadvantage is that this method differs sufficiently from what actually occurs in clinical practice, since cells in the proliferative phase are directly placed in the medium which lacks nutrients. In this condition, the reaction may be different from what happens in reality. Hence, in the present study we used the second method, which more closely replicates the actual clinical scenario.<sup>20</sup>

In this study, 0.4% trypan blue exclusion staining technique was used as it is quick, easily performed and distinctively singles out the nonviable cells from viable cells. The reactivity of the stain trypan blue 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. Thus, all the cells which exclude the dye are viable.

According to the results, tap water maintained the least number of viable cells followed by gatorade, saline,

coconut water and egg albumen, while milk and lens solution demonstrated higher values. Amongst them, the low fat milk group had the highest percentile of viable cells followed by the contact lens solution group upto 6 hours; whereas contact lens solution showed highest viability upto 24 hrs. Khademi et al. in 2008<sup>20</sup> have compared milk and egg white solutions as media for storing avulsed teeth and have shown that when teeth were stored in egg white for 6 to 10 hours, they had a better incidence of repair than those which were stored in milk for the same time period. Sousa et al. in 2008<sup>25</sup> have microscopically analyzed the human periodontal ligament attached to the extracted tooth after one hour of extraoral time and compared milk, egg white, and artificial saliva. They also state that teeth stored in milk and egg white were similar in terms of collagen fibre organisation and cell numbers. Artificial saliva had an inferior result. It was suggested that egg white, can be the perfect medium for storing avulsed teeth. In our study, egg white maintained viability for 2 hrs and at the end of 2 hrs the difference between the ability to maintain the viability between water and egg white was not significant. This indicates that egg white can be used as a suitable storage medium for a period of 2 hrs. Beyond this, the viability of PDL is not well maintained. We assume that this decline in the number of viable cells could be due to the deterioration of egg white when exposed to room temperature.

Tap water has shown the least desirable results. Though it protects the tooth from dehydration, for being a hypotonic medium, it causes rapid cellular lysis of the PDL, similar to a dry storage.<sup>7,8,21,26,27,28</sup> In our study, tap water was the least preferred storage media as compared to the other media. However, at 6 hr and 24 hr interval, the statistical difference between water, coconut water and egg white was insignificant.

Coconut water is a natural drink. The mineral composition of coconut water is similar to intracellular fluid more closely than extracellular plasma. Potassium, calcium, and magnesium are predominantly found. Sodium, chloride, and phosphate are also found in lower concentrations. It is a hypotonic solution that is more acidic than plasma. Its specific gravity is approximately 1.020, comparable with blood plasma. Coconut water can be given as a means of rehydration to patients with potassium deficiency because it is readily acceptable to the human body. Coconut water obtained from the fruit of coconut palm is grown in more than 93 countries around the world, most commonly in South Asian countries, South East Asian countries and Pacific nations. It also has a significant presence in Jamaica and Mexico. It is also available in tetra packs in most of the supermarkets. Thus, coconut water which is easily available, natural and hygienic can be advocated as a good transport medium for avulsed teeth.

Gopikrishna et al.<sup>30</sup> in 2008 found that coconut water was superior to Hanks Balanced Salt Solution (HBSS), propolis or milk in terms of maintaining viability of PDL

cells after avulsion and storage. A study done by H Geeta et al<sup>16</sup> in 2008 also states that coconut water performed better than milk and propolis, and thus they concluded that coconut water which is readily available and effective can be used as a suitable storage media. Moeira et al<sup>29</sup> in 2009 suggested milk had the greatest capacity to maintain cell viability. Coconut water was significantly inferior at maintaining cell viability compared to other media like milk, coconut water with sodium bicarbonate and saline. In our study, for 1, 2 and 4 hour intervals, coconut water showed insignificant values as compared to milk and egg white, which means that coconut water can be used as a suitable storage medium. The composition of the storage media would be of minor importance for cell viability and membrane integrity after storage of up to 3 hours if the media had a physiologic osmolality. Hypotonic media seemed to potentiate the damaging effect of bacterial contamination.<sup>35</sup>

Milk is an inexpensive dairy product which is commonly consumed and easily available to the general population. Several investigators like Blomlof and Otteskog 1980<sup>20</sup>, Marino et al. 2000,<sup>21</sup> Lekic et al. 1998,<sup>22</sup> Patil et al. 1994<sup>23</sup> compared milk with several other storage media and found that milk was superior to the others in maintaining the viability. Physiologic osmolality is an important characteristic for the transport media to possess, in order to maintain PDL cell viability.<sup>24</sup> Studies have also shown that, in cool conditions, cells have a higher percentage of viability than at room temperature, as cooler temperatures decrease cell swelling, increase cell viability, and improve recovery, all of which promote wound healing. Also, not all types of milk are equally effective as storage media. Some evidence supports the use of cold milk as a provisional storage medium for avulsed teeth. Avulsed teeth stored in chilled milk for up to 1 hour can maintain sufficient number of viable periodontal ligament cells to support replantation of the tooth and the possibility of periodontal ligament healing.<sup>31,33</sup>

Commercially available milk is pasteurized, thus inactivating enzymes that are potentially harmful to the PDL. Regular pasteurised milk has a short shelf life requiring refrigeration, which makes it less readily available at the trauma site. Thus long shelf-life milk having identical composition, pH, and osmolality to regular milk with a storage capability of 6 months without the need for refrigeration has gained more acceptance.<sup>34</sup> Therefore milk, cold or otherwise, can be used as a storage medium of choice for extended extra-alveolar storage (1 to 6 hr).

As with many in-vitro studies, limitations and variability often exist. Milk, although superior to water and saliva as a storage medium, has not shown to have the capacity to reconstitute lost cellular metabolites. It also doesn't possess the ability to maintain morphological integrity of the periodontal ligament cells. Milk only prevents cell death instead of reviving normal morphology and the ability to differentiate and mitose.<sup>35</sup>

Gamsen et al. showed that milk maintains the osmotic pressure for periodontal ligament cells, but it does not possess the ability to reconstitute depleted cell metabolites and restore viability.<sup>32</sup> It had been recommended that, even if an avulsed tooth has been kept in physiologic media like saline and milk immediately after avulsion accident, the teeth should still be soaked in HBSS for 30 min before they are replanted into the sockets, because neither saline nor milk can revive depleted cell metabolites.<sup>36</sup>

Normal Saline is the commonly used term for the solution of 0.9% w/v of NaCl, with an osmolality of 300mOsm/L. It is known as rehydrating fluid when used intravenously. Various investigators have used saline as a storage media for transplants.<sup>42</sup> Alacam et al. 1996<sup>40</sup> concluded from his study that sterile saline solution would likely be a poor storage media because it lacks metabolically essential ions such as Ca<sup>++</sup> and Mg<sup>++</sup>, and it does not provide glucose to cells, causing extensive destruction to them. Saline was used by Andreasen in 1980<sup>43</sup> in monkeys as a storage medium for mature permanent incisor replants to analyse the topography of surface and inflammatory root resorption in these replants. These authors believed, that saline could maintain the vitality of the periodontal membrane. Lindskog et al. 1982<sup>44</sup> 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. Huang S C et al. 1995<sup>45</sup> conducted a study with 4 different storage media: K-mart contact lens solution, Alcon opti-free contact lens solution, milk and Saline and concluded that saline was superior to both the contact lens solutions in its efficiency to maintain PDL cell viability. The findings in the above studies are in agreement with the present study which also showed that as time advanced, the number of viable cells decreased in number in saline. A number of individuals in the present era use contact lenses. The most popular types are the multipurpose solutions. These solutions have been approved as a single bottle system for cleansing and disinfecting contact lenses. According to the manufacturers, these solutions are designed to store the lenses, which are then placed into the eye without the need for a prior rinse with water or saline, implying that they are not detrimental to the sensitive ocular tissues.

According to Sigalas, et al. 2004<sup>38</sup> contact lens solution preserved significantly greater number of viable cells than tap water both at ice and room temperature and significantly greater cells than Gatorade® at room temperature. So it is suggested from the study, that the contact lens solution can be used as a short term storage medium when other solutions cannot be availed of. In the present study, the viable cells in contact lens solution were estimated against time, and the findings are parallel to the study by Sigalas et al. 2004,<sup>38</sup> demonstrating a high percentage of viable cell count till 4 hours time period, and as time progressed the value reduced.

Exarticulation occurs mostly within an athletic environment. Oral rehydration fluids such as Gatorade®,

are frequently available in these settings. According to exercise physiology laboratory at the sports science institute, Gatorade® thirst quencher has a pH of 3 and osmolality ranging from 280 to 360 mOsm/L.

In the present study, among the storage media compared, Gatorade® was found to have less mean viable cell count. Harkacz et al. 1997<sup>41</sup> revealed that Gatorade® did not prove to be suitable as a potential temporary storage media for avulsed teeth. Chamorro et al. 2008<sup>37</sup> showed that Gatorade® contains fructose and glucose polymers as an energy source for the cells. But in contrast, Gatorade® has an apoptotic trigerring effect in human PDL cells as it causes mild cell membrane damage due to the low pH which is not conducive to cell growth or survival. Due to the above reasons Chamorro et al. 2008<sup>37</sup> and Olson et al. 1997<sup>39</sup> suggested Gatorade® as an unsuitable storage media. Blomlof and Lindskog 1982<sup>22</sup> through their study concluded that cell survival was more favourable in physiologic than in hypotonic osmolalities. Blomlof, Otteskog and Hammarstrom 2009<sup>45</sup> correlated cell leakage with osmolality, where cells stored in hypotonic solutions leaked more than cells in physiologic solutions. In this study, among all the solutions compared, Gatorade® preserved least % of cells at 1 hr (33%) which was in agreement with the findings of the above studies. Thus, Gatorade® can be used as a short term storage medium when other solutions cannot be obtained.

Trypan Blue staining technique has been used to assess the cell viability in most of the studies including the present study. The health status of viable periodontal ligament cells is critical in preventing resorptive sequelae of post replantation and the Trypan blue stain used here only assesses the vitality of the cell and not actual physiologic health or metabolic capabilities of the cell, restraining the study. Thus, more auxiliary studies are required in this regard. There is also the possibility of intra observer bias, by the observer in counting the viable periodontal ligament cells. Despite the in-vitro limitations and variability encountered in this study, milk demonstrated promising results in terms of maintaining periodontal ligament cell viability for a prolonged period of 6 hrs and hence, poses to be the most reliable and a gold standard storage media.

## CONCLUSION

Immediate replantation is the best treatment for an avulsed tooth, provided the tooth has viable PDL cells at the time of replantation. Storage media aid in preserving the viability of the periodontal ligament cells when immediate replantation is not possible. This study evaluated the post-traumatic periodontal ligament cell viability following storage in different storage media at different time intervals.

From the present study, it is concluded that:-

- There is a steady descent in the number of viable cells in all the experimental storage media as time passes.

- Low fat milk is the most effective medium for maintaining the viability of PDL upto 6 hours, whereas contact lens solutions maintain the highest number of viable cells after 6 hours upto 24 hours.
- Milk can also be used as a storage medium, but for a short period of about 6 hours after which its efficacy declines.
- Low fat milk stands first in maintaining the PDL cell viability for a longer duration than medium and high fat content milk.
- Egg white is a suitable storage medium but for a short period of about 2 Hours, after which its efficacy declines.
- The efficacy of coconut water can be comparable to that of milk for a period of upto 4 hrs.

**Recommendation:** Low fat milk is the most preferred medium for maintaining PDL cell viability.

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