ORIGINAL RESEARCH

Comparative Evaluation of Maintenance of Cell Viability of an Experimental Transport Media "Ringer's Lactate" with Dextrose Normal Saline ORS Egg White and Infant Milk Formula for Transportation of an Avulsed Tooth

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Abstract

Background and objective: The viability of the periodontal ligament (PDL) cells on the root surface of the avulsed tooth determines the prognosis of the replanted tooth, which in turn is determined by a suitable transport medium in which the tooth was stored. The aim of the present study is to evaluate and compare the effectiveness of Ringer's lactate (RL) as a storage medium for an avulsed tooth in maintaining the PDL cell viability with dextrose normal saline (DNS), oral rehydration salt (ORS), egg white (EW), and infant milk formula (IMF).

Materials and methods: A total of 85 freshly extracted human teeth were divided into five experimental groups and two control groups. The positive and negative controls corresponded to 0-minute and 8-hour dry time, respectively. The experimental teeth were stored dry for 30 minutes and then immersed in one of five experimental media (RL, DNS, ORS, EW, and IMF) for 45 minutes. The teeth were then treated with collagenase type III and trypsin for 10 minutes. The number of viable PDL cells was counted with a hemocytometer and analyzed.

Results: Statistical analysis showed that IMF, RL, and EW had no statistically significant differences among them in maintaining the viability of the PDL cells but were significantly better than DNS. No statistically significant difference between RL, EW, and ORS in the number of viable PDL cells.

Conclusion: Infant milk formula (IMF), RL, and EW showed similar results within the parameters of the study; they can be used as alternative storage media for avulsed teeth. DNS showed poor results, and ORS could serve as short-term storage media if the other solutions are not readily available.

Clinical significance: The search for an appropriate storage media with favorable pH and osmolality along with easy availability is the basic thought behind this study.

Keywords: Avulsed teeth, Dextrose normal saline, Egg White, Infant milk formula, ORS, Ringer's lactate, Storage medium.

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Introduction

Tooth avulsion or exarticulation is an extreme form of traumatic dental injury that depicts the total expulsion of a tooth from the alveolus. It holds to be 0.5–16% of all dental trauma-related injuries to permanent anterior teeth and about 7–13% in the primary dentition.[1,2]

Among incisors, maxillary ones are frequently involved, especially at the age of 7–9 years, during the eruption of these incisors. When dental exarticulation occurs, there and then, the replacement of the tooth in the empty alveolar socket is the ideal course of action for the survival of PDL cells. However, instantaneous reimplantation is seldom achieved,[3] due to multiple associated factors such as consciousness of the patient, no familiarity with first aid regarding tooth avulsion, consent of patient and family members, and fear issues along with apprehension in people around the scene of the accident.[4] It was initially thought that the success of replantation is primarily associated with the rapidity with which the tooth is replaced in the socket, that is, the briefer the time outside the socket, the better the prognosis, but this concept has been transformed with time and investigators now state about storage media as a major influencing factor than extraoral time, as dry time even for the shorter period showed poorer prognosis than prolonged storage in a suitable storage media.[5]

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A storage medium is said to be a physiological solution that closely replicates the oral surroundings and helps to conserve the viability of PDL cells following exarticulation.[6] Various types of storage media for avulsed teeth have been studied; however, we still seek an ideal storage media which can overcome the limitations of the previously used ones.[7]

It is of interest to identify an effective, readily available, and economical storage medium that can conserve the viability of PDL cells resulting in a high percentage of successful replantation. Thus this study was executed with the goal of assessing the effectiveness of RL as a media of storage for an avulsed tooth to preserve the viability of PDL cells along with other storage media such as DNS, ORS, EW, and IMF.

Materials and Methods

A total of 85 freshly extracted human premolar teeth with closed apices, having normal periodontal health, which were extracted atraumatically, were chosen for this study. Patients selected were below 30 years of age. Traumatically extracted teeth, teeth with periodontal issues or caries or any developmental anomaly, or those damaged while handling after extraction were excluded from the study. Following extractions, the premolars were held from the coronal region with the help of forceps. The PDL cells that might have been damaged were scraped off from the coronal 3 mm of the PDL with a sharp curette.

Preparation of Storage Media

Ringer's lactate (RL), DNS, ORS, and IMF were commercially procured and prepared freshly as per the manufacturer's instructions for each sample. Eggs were commercially procured and were then thoroughly washed. A little part of the eggshell was cracked to allow the white to drain into the sterile test tube through the crack hole.

Method of Storage of Teeth

After tooth preparation, random assignment of samples was done to one of the five experimental storage media ranging from groups I to V, that is, RL, DNS, ORS, EW, and IMF, with 15 samples per group. After a dry time of 30 minutes in all the experimental groups, including the time for curetting, subsequently, they were placed in one of the five media of storage for a duration of 45 minutes.

Two control groups were made with five teeth each. Teeth in the positive control (group VI) were assayed immediately for cell vitality without any dry time or being stored in any storage media. Teeth in the negative control (group VII) were kept extraoral with a dry time of 8 hours following no storage media time and later assayed for cell vitality.

Isolation of PDL Cells

Each tooth sample, subsequent to extraoral time and storage media time, was delicately washed in phosphate buffer saline (PBS) to get rid of blood and debris clinging to the radicular part of the teeth. Each PBS-rinsed tooth sample was kept in a sterile 15 mL Falcon tube for 10 minutes. The tubes contained 1 mL of enzyme solution (collagenase type III and trypsin) to enzymatically isolate PDL cells. For the last 2–3 minutes of immersion, the solution was shaken with the help of a micropipette to detach the cells. After 10 minutes of enzyme treatment, the teeth were removed from the solution. In a separate microtube, 1 mL of enzyme solution was taken into which 10 µL of fetal bovine serum was mixed. Centrifugation of the microtube was done at 90 rpm for 4 minutes.

Cell Viability Assay

The loss of membrane integrity was assessed using trypan blue (0.4% (w/v) in a 1:1 ratio (100 µL of solution with 100 µL of dye), also called the dye exclusion test. Enumeration of viable and nonviable cells was carried out using Neubauer's counting chamber, which was viewed on a light microscope at 10× magnification.

Results

Statistical analysis was carried out using a one-way analysis of variance, and multiple comparisons were made using the *post hoc* Scheffe test. The significance level was set at the 5% limit. The cells which maintained the integrity of the cell membrane were considered viable. The mean of viable cells obtained in various groups is presented in Table 1. Amongst the storage media, the maximum number of mean viable PDL cells was shown by teeth stored in IMF, followed by RL, EW, ORS, and DNS, respectively. Figures 1 and 2 compare the mean vital cells in each group. The outcomes of the analysis of variance (ANOVA) showed differences among the groups, which were statistically significant ($p \leq 0.05$) and summarized in Table 2. Table 3 represents three points:

- Statistically significant difference between positive and negative control group with that of experimental groups ($p \leq 0.05$).
- Statistically significant difference between IMF and ORS, IMF and DNS, EW and DNS, and RL and DNS ($p \leq 0.05$).
- No statistically significant difference between DNS and ORS, ORS and RL, ORS and EW, RL and EGG, RL and IMF, and EGG and IMF.

Table 1: Summary statistics of the mean number of viable cells

Group	*No of samples*	*Mean*	*Standard deviation*
RL	15	320.00000	23.400244
DNS	15	287.46667	19.141827
ORS	15	298.06667	15.021255
EW	15	316.20000	22.722550
IMF	15	341.80000	19.896877
Positive control	5	521.80000	22.554379
Negative control	5	1.00000	1.732051

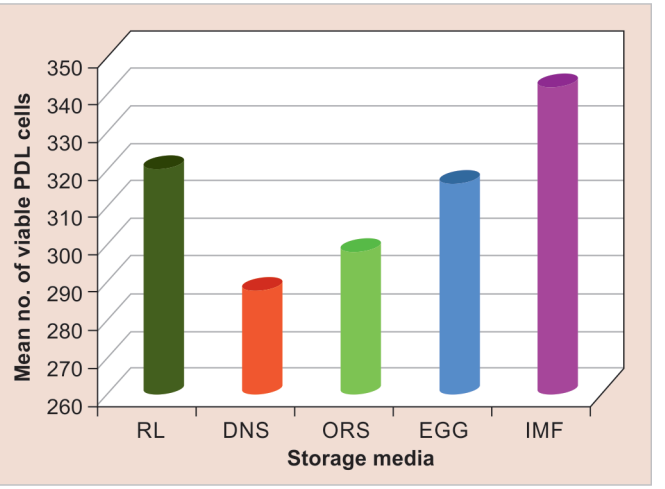

Fig. 1: Comparison of five experimental groups with respect to the mean number of viable PDL cells

Discussion

The principal cause for reimplantation failure in traumatically avulsed teeth is the absence of viable PDL cells. The direct correlation between the survival of a reimplanted tooth and the number of viable PDL cells was first addressed by Hammer in 1955. The study by Andreasen and Hjorting-Hansen mentions immediate replantation as a treatment modality for avulsed teeth.[8,9] However, if the same is not possible, teeth must be stored in a suitable storage media having the ability to maintain the viability of periodontal cells till the time definitive treatment is rendered.[10]

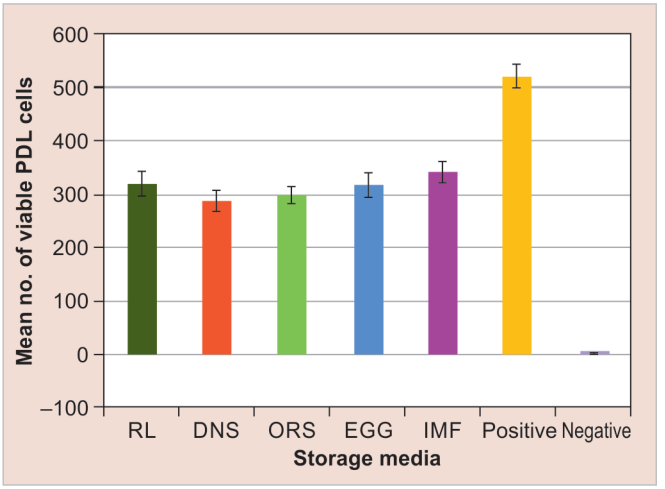

Fig. 2: Comparison of seven groups with respect to the mean number of viable PDL cells. The error bar represents the standard deviation

Studies report that a pH of 6.6–7.8 and an osmolality of 230–400 mOsmol/kg are required to conserve the viability of PDL cells.[11]

Ringer's lactate (RL) solution has the tonicity equivalent to that of blood and is intended for intravenous administration. It was one of the first laboratory solutions of salts in water, which showed to prolong the life of tissues removed from the body. According to Bharatha et al. RL can be used as a feasible storage media for exarticulated teeth due to its good osmolality, cost-effectiveness, and better availability.[12]

Dextrose normal saline (DNS) is a parenteral solution available in various concentrations. In the present study, 5% DNS, which is commonly available in all pharmacies, as ready to use a solution containing 5% of dextrose and 0.9% sodium chloride, thus providing a source of water, carbohydrate, and electrolytes.

Oral rehydration solution (ORS), a combination of glucose and vital salts used for treating dehydration, has been observed to provide physiological pH and osmolality, which makes it suitable for cellular growth. Ease of availability and being inexpensive are

Table 2: Comparison of seven groups with respect to the number of viable cells by one-way ANOVA test

	Sum of squares	*Degree of freedom*	*Mean square*	*F-ratio*	*p-value*
Between groups	727756.510	6	121292.752	307.447	<0.0001 Highly significant
Within groups	30772.267	78	394.516		
Total	758528.776	84			

*Significant ($p \leq 0.05$)

Table 3: Pair-wise comparison of groups with respect to the mean number of viable cells by Tukey's honestly significant difference multiple *post hoc* test

Comparison	*Mean difference*	*p-value*	*Significance*
Positive vs group DNS	234.333	<0.001**	Highly significant
Positive vs group ORS	223.733	<0.001**	Highly significant
Positive vs group RL	201.800	<0.001**	Highly significant
Positive vs group EW	205.600	<0.001**	Highly significant
Positive vs group IMF	180.000	<0.001**	Highly significant
Negative vs group DNS	–286.466	<0.001**	Highly significant
Negative vs group ORS	–297.066	<0.001**	Highly significant
Negative vs group RL	–319.000	<0.001**	Highly significant
Negative vs group EW	–315.200	<0.001**	Highly significant
Negative vs group IMF	–340.800	<0.001**	Highly significant
Group DNS vs group ORS	–10.600	0.904	Not significant
Group DNS vs group RL	–32.533	0.005	Significant
Group DNS vs group EW	–28.733	0.023	Significant
Group DNS vs group IMF	–54.333	<0.001**	Highly significant
Group ORS vs group RL	–21.933	0.181	Not significant
Group ORS vs group EW	–18.133	0.405	Not significant
Group ORS vs group IMF	–43.733	<0.001**	Highly significant
Group RL vs group EW	3.800	1.000	Not significant
Group RL vs group IMF	–21.800	0.187	Not significant
Group EW vs group IMF	–25.600	0.065	Not significant

**, highly significant $p < 0.01$

other beneficial properties.[13–15] Mahesh et al. compared electrol solution, RL, coconut water, and ORS liquid to conclude that RL showed maximum PDL cell viability at various time intervals, which was followed by coconut water, electrol solution, and ORS-L.[16]

Egg white (EW) protein ovalbumin contains all the nutritionally indispensable amino acids along with vitamins and water. It also prevents bacterial contamination due to the presence of lysozyme, which can destroy bacterial cell walls.[17–19] Taking these properties as well as eases of availability into consideration, EW was selected as the storage media for comparison.

Milk has the ability to maintain human PDL cell viability[20] and is accepted as a suitable transport medium by the American Association of Endodontists for avulsed teeth.[21] Pasteurized milk, as well as long shelf-life milk both, were seen to be more efficient in conserving the human periodontal cell viability than Save-A-Tooth at 8 hours, according to Marino et al.[22] To find a substitute for milk that is equally efficacious but more convenient to transport and maintain within easy reach, we compared the milk substitute—IMF.

Teeth extracted for any reason can simulate avulsed teeth, as shown in previous research.[7] As in the study done by Martin and Pillegi,[23] in the present study, the extraoral time considered was 30 minutes to mimic the typical clinical scenario of dry time, after which the teeth were placed into a storage medium. The initial 30 minutes is the time when maximum damage occurs to the PDL cells, with some cells left viable for assessment. So this duration was chosen for the study.[24]

The time duration of 45 minutes was chosen for the storage of sample teeth in experimental storage media in this study as taken by similar previous studies to facilitate comparison.[10,23,25] In the current study, enzymatic isolation of the cells using collagenase type III and trypsin was done from the PDL.[7] Procedure facilitated speedy cell retrieval along with preserving cellular integrity. Trypan blue dye exclusion staining method was selected as it is simple and economical, along with an uncomplicated technique that accurately differentiates nonviable cells from viable cells. It is based on the fact that the chromophore within the dye is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells are considered viable, which excludes the dye.[14]

In the present study, the positive control had no dry time resulting in the highest number of mean viable cells. Amongst the storage media, milk displayed a maximum number of mean viable cells followed by RL, EW, ORS, and DNS. Negative control, as it was left to dry for 8 hours, displayed the least number of viable cells. This was because of the complete loss of PDL cell viability due to dehydration. The difference between the experimental group with the positive and negative control group was statistically significant.

Among the experimental groups, IMF, RL, and EW showed no statistically significant differences in conserving the viability of PDL cells but were significantly better than DNS. There was no statistically significant difference between DNS and ORS, but individually IMF was significantly better than ORS. No statistically significant difference was seen between RL, EW, and ORS in the count of viable PDL cells.

Very few studies have reported the relationship between the experimental media we used, so comparisons are done wherever possible. Milk has neutral pH and physiologic osmolality with no or low bacterial contamination, has essential nutrients and growth factors for cell survival, and also has the advantage of low cost and ease of availability.[26,27] Several authors who have considered milk to study the viability of PDL cells have shown 70–90% of survival rates and fewer root resorptions for up to 72 hours.[25,28–30] Pearson et al.[31] compared regular pasteurized whole milk with various milk substitutes, such as reconstituted powdered milk, evaporated milk, and two baby formulas (similac and enfamil). They stated that powdered form enfamil having 18 months of shelf life, is a more convincing storage medium for avulsed teeth than whole pasteurized milk for a time of 4 hours.[32] Correlating with the results of the already done studies, our study proved that milk is a promising storage medium for avulsed teeth. In addition, we used IMF (lactogen), supplied as milk powder, having an extended shelf life along with no requirement for special storage.

Lactated Ringer's solution, which has similar concentrations of potassium and calcium as found in normal blood plasma, was also used in the present study. Buffa et al.[33] studied the efficiency of phosphate-buffered saline, sterile tap water, normal saline, and RL on the viability of canine embryonic fibroblasts. They showed that RL was not responsible for any major fibroblastic injury, but normal saline and sterile tap water showed mild to severe cytotoxic effects on wound healing in an *in vitro* study. A similar observation was made in the present study, RL was significantly better than dextrose in normal saline (DNS). In the current study, it was observed that RL is similar to IMF in its capacity to maintain cell viability as a storage medium for avulsed teeth. This capability might be attributed to the composition, physiologic pH, and osmolality.

Sousa et al.[30] observed that EW is comparable to milk in maintaining the histological characteristics and PDL cell viability. Moreover, Rozenfarb et al.[34] observed no significant difference between minimum essential medium, egg albumen, and milk; all were superior to saliva. In addition, Ahangari et al.[35] reported no significant difference between milk and EW in conserving the viability of PDL cells. The same findings were seen in the present study. In contrast, Khademi et al.[17] reported no significant difference between HBSS and EW in an *in vitro* study at different storage times. Both were more efficient than water or milk. In the current study, milk and egg showed no significant difference.

Subramaniam et al.[14] found that oral rehydration solution-liquid is as effective as HBSS in maintaining the PDL cell viability, and it also was superior to milk. Rajendran et al.,[7] in their study, summarized that the ability of Ricetral, an oral rehydrating solution, to retain PDL cell vitality was similar to HBSS, and both these solutions were better than milk. In contrast, our results were in contrast to the studies by Subramaniam and Rajendran et al. In the present study, ORS was found to be similar to RL and EW in its efficacy as a storage medium. Also, the results of ORS and DNS were comparable when the mean numbers of viable cells were studied.

In this study comparison of the mean number of viable PDL cells was done among the studied storage media, and IMF showed better results, followed by RL, EW, ORS, and DNS. The favorable results of IMF can be attributed to its physiological properties, including pH and osmolality compatible with those of the cells from the PDL. In addition, the presence of nutritional substances, such as milk protein, carbohydrates, vitamins, and minerals.[10,36]

Limitations and variability are a part of in vitro studies that do exist with the present study design as well. In our study, the extractions were performed by different clinicians, which might have induced variable trauma during the actual extraction. This could affect viable PDL cell counts. Trypan blue used in this study to assess cell viability only assesses the vitality of the cell and not the actual physiologic health or metabolic capabilities of the cell. Further, it does not differentiate between necrotic and apoptotic cells. These storage solutions mainly deal with issues such as drying

and cell metabolite depletion. However, other major concerns, such as the regenerative capability of viable fibroblasts, root resorption, and contamination, should also be taken into account. So using agents to reduce root resorption and antibacterial component to handle possible contamination should be considered.

Conclusion

The search for an appropriate storage media is still desirable, having all the required characteristics, that is, the one capable of conserving the viability of the PDL along with physiological pH and osmolality. Other important characteristics include antioxidant properties, no or minimal microbial contamination, ease of accessibility at accident sites, homes, schools, hospitals, and dental offices, and, most importantly, low cost. Based on our results, it could be stated that:

- As IMF, RL, and EW, within the parameters of the study, revealed comparable results, it is concluded that RL can be a suitable alternative as a storage media for avulsed teeth.
- Dextrose normal saline (DNS) showed poor results among the studied experimental media, probably because of its low pH and hypertonic osmolality.
- Oral rehydration solution (ORS) can be considered as a short-term storage medium in case other media are not readily available.

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