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Original Article

Dentistry Section

Comparative Evaluation of The Efficacy of Aloe Vera Gel with Milk and Hank's Balanced Salt Solution in Maintaining the Viability of PDL Cells in Avulsed Teeth

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ABSTRACT

Introduction: Various storage media have been investigated due to their ability to maintain the viability of the Periodontal Ligament (PDL) cells, thus permitting longer extra-alveolar periods prior to replantation of avulsed teeth.

Aim: To assess the efficacy of a newer storage medium, Aloe Vera Gel in comparison with milk and Hank's Balanced Salt Solution (HBSS) in maintaining the viability of PDL cells in simulated avulsed teeth.

Materials and Methods: The present in-vitro study was performed on 40 freshly extracted human premolars with intact crown and closed apices, extracted for orthodontic reasons. They were randomly assigned to one of the three storage medium groups (N=10 each) and a positive and negative control (N=5 each). The positive and negative controls corresponded to 0 minute and an eight hours dry time respectively. The teeth in the experimental group were dried at room temperature for 30 minutes followed by 45 minutes immersion in one of the three experimental media. After drying and soaking, the root surface of each tooth was treated with an enzymatic solution containing 1 mL of Collagenase Type II and Dispase Type I in phosphate buffer saline (PBS). The cells were then labelled with 0.4% Trypan blue and counted using a haemocytometer under a light microscope at 10x magnification. Kruskal Wallis H-test was used to analyse the mean difference in the number of viable cells among the five groups.

Results: The teeth stored in HBSS demonstrated the highest number of viable PDL cells followed in rank order by milk, positive control, aloe vera and negative control. There was no significant difference in the number of viable PDL cells between HBSS and milk.

Conclusion: From the results of the present study it can be concluded that aloe vera may be better than negative control to maintain PDL cell viability but not more than HBSS and milk.

Keywords: Immediate transplantation, Periodontal ligament, Storage media

INTRODUCTION

Avulsion, the complete expulsion of the tooth from the socket, is considered as the most serious form of traumatic tooth injury [1]. Though ideal treatment of choice is immediate replantation, it is not always possible due to other life-threatening injuries, complex injury to the socket, or lack of awareness about replantation procedures. Periodontal ligament repair and reduction of possibility for root resorption are the favourable outcomes of immediate transplantation. The ill effects of the extra-alveolar period can be prevented by the time the tooth remains out of the socket and immersing the avulsed tooth in a suitable transport medium. However, depending on the time of storage and the temperature and characteristics of the transport medium, harmful effects such as ankylosis and root resorption may still occur [1].

Different types of moist storage media have been experimented. Hank's Balanced Salt Solution (HBSS) which was commonly used in biomedical research to protect different cell types is considered as the gold standard as it has the capacity to maintain the viability of cells of periodontal ligament without any morphological distortion [2] and has been shown to be effective for preserving avulsed teeth for extended periods of time [3]. Other solutions like Viaspan and Euro-Collins, culture media, saline, natural products like saliva, water, bovine milk and its variations, green tea, propolis, Morus rubra (red mulberry), coconut water, egg white; rehydrating solutions, like Ricetral and Gatorade, and even contact lens solutions have also been investigated [1]. It has also been noticed that the lack of availability and high cost limits the use of majority of these media. Hence the search for products commonly available at the site of injury has become the need of the hour. However, all the ideal requirements were not met by the presently used media and quest still continues for one that is able to overmatch the drawbacks of these materials [4].

Thus, the present study aims at evaluating the efficacy of HBSS and two other easily available natural products like Aloe vera gel and milk which are used as a transport medium for teeth following an avulsion, by using a collagenase-dispase assay and trypan blue dye exclusion test.

MATERIALS AND METHODS

The present in-vitro study was done in the Department of Paediatric Dentistry, Amrita School of Dentistry, affiliated to the Dental Council of India, study was done in 2017 (V.12017/51/2002-DE). The biochemical analysis was performed at Unibiosys Biotech Research Laboratory, Kochi Kerala, India. Institutional ethical clearance was obtained from the Institute's Ethical Committee before conducting the study (Approval ref: 022/TPRC/2016).

Sample selection

The sample size was calculated based on the mean and standard deviation of the variable, viable cell count in milk and aloe vera obtained in the study by Sharma M et al. in 2015 [5]. With 95% confidence interval and 90% power, the minimum sample size needed was estimated to be 10 samples per group.

Hence the sample size of the present study included 40 human premolars indicated for orthodontic extraction. These teeth had intact crown and closed apices, devoid of enamel defects, dental caries, restorations and periodontal disease. Immediately after extraction, the teeth were grasped at the coronal region with

tweezer. Trauma caused during extraction could have damaged the PDL cells around the coronal region. Hence, removal of these cells was done by scraping 3 mm around the coronal portion of the root. Extractions were carried out by a single operator, as atraumatically as possible.

Sample Grouping and Storage

The three experimental storage media used were commercially available HBSS (HiMedia Mumbai, India) pasteurised low fat cow's milk and freshly extracted 100% aloe vera gel. The teeth were randomly assigned to one of the three storage medium groups with 10 samples per group and a positive and negative control consisting of five samples each. The teeth in the experimental group were dried at room temperature (i.e. 25°C-26°C) for 30 minutes, including the time needed for curetting.

This was followed by a 45-minutes immersion in one of the experimental media. This time period was selected from the studies which had followed the same methodology [6-8].

Preparation of Aloe Vera extract

The Aloe Vera leaf was thoroughly washed and placed in 70% ethanol alcohol. Following strict aseptic method, the leaf was slit opened and the viscous gel was scrapped from the inner portion of the leaf with the help of a scalpel, followed by homogenising and filtering using a 0.45 µm filter mesh.

Each experimental medium was then taken in a container and the extracted teeth were completely immersed in it. The experimental groups were designated as,

Group I-HBSS

Group II-Milk

Group III-Aloe vera

Group IV-Positive control

Group V-Negative control

The positive control group constituted teeth that were neither dried nor stored in any solution, but assayed instantly for cell viability. The negative control teeth were bench- dried for eight hours, and without placement in any medium, was directly assayed.

Isolation of Cells

The blood and debris which adhered to the roots of each sample were mildly washed with Phosphate Buffer Saline (PBS). A collagenase-dispase assay was used for the extraction of cells, enzymatically from the periodontal ligament.

Collagenase Type II (HiMedia, Mumbai, India) and Dispase Type I (HiMedia, Mumbai, India) was used in the study.

The enzyme solution for the experiment was composed of (in 5 mL):

Collagenase Type II (lyophilized powder, 152 U/mg) 1 mg

Dispase Type I solution (1.5 g per 100 mL) 0.8 mL

PBS 4.2 mL

Each PBS-rinsed sample was immersed in 1 mL of the enzyme solution for 10 minutes in a sterile 15 mL Falcon tube. The solution was agitated using a micropipette for the last 2-3 minutes of immersion, to allow detachment of cells. The teeth were then withdrawn from the solution and 1 mL of the solution was pipetted to a microtube. Ten microliters of fetal bovine serum (HiMedia, Mumbai, India) was added to it and centrifuged at 90 g for four minutes. The supernatant was removed, and the pellet was dissolved in 1 mL of PBS [4].

Staining and Counting of Cells

Staining was carried out using 0.4% (w/v) Trypan blue (HiMedia, Mumbai, India) in a 1:1 ratio (100 µL of solution with 100 µL of dye). After 10 minutes, 10 µL of the staining solution was taken and the number of viable (visible as clear cells as they do not take up the stain) and nonviable cells (cells that absorb the stain) was counted under a light microscope with a haemocytometer at 10x magnification [4].

Compliance with Ethical Standards

- Research involving human participants and/or animals- This article does not contain any studies with human participants or animals performed by any of the authors.
- Informed consent- Institutional Ethical Clearance was obtained for this study; however, human subjects were not a part of this work.

STATISTICAL ANALYSIS

SPSS software version 20.0 was used for the statistical analysis. The mean difference in viable cell count among the five groups was analysed by Kruskal Wallis H-test. The significance level was standardised to 0.001. Mann-Whitney U-test was used to compare the level of significance between individual groups. p-value<0.05 was considered significant.

RESULTS

Viable cells with intact cell membrane were observed as clear cells as they did not absorb the stain and the non-viable cells absorbed the stain due to the compromised cell membranes.

Group I (HBSS) showed the maximal number of mean viable cells among all the groups (921.40±608.438) followed by Group II (812.70±449.170). The ability of Group III (241.00±194.572) to preserve viable cells was lower than Group I, Group II and Group IV (716.60±53.257). Group V showed the least number of viable cells (156.40±48.418). There is a significant difference in the mean number of viable cells among the five groups tested (p<0.001) [Table/Fig-1].

Groups	N	Mean No. of viable cells	Std. Deviation	p value
I	10	921.40	608.438	
II	10	812.70	449.170	
III	10	241.00	194.572	<0.001
IV	5	716.60	53.257	
V	5	156.40	48.418	

[Table/Fig-1]: Indicating the p value for mean number of viable cells

Statistically significant differences were found between Group I and Group III, and also between Group II and Group III. However, no significant difference existed between Group I and Group II [Table/Fig-2a, b, c].

Groups	Mean±SD	Asymp. Sig. (two-tailed)	Significance
Group I	921.40±608.438	0.94	Not significant
Group II	812.70±449.170		

[Table/Fig-2a]: Comparison between Group I and Group II by Mann-Whitney U test.

Groups	Mean±SD	Asymp. Sig. (two-tailed)	Significance
Group I	921.40±608.438	0.001	Significant
Group III	241.00±194.572		

[Table/Fig-2b]: Comparison between Group I and Group III by Mann-Whitney U test.

Groups	Mean±SD	Asymp. Sig. (two-tailed)	Significance
Group II	812.70±449.170	0.001	Significant
Group III	241.00±194.572		

[Table/Fig-2c]: Comparison between Group II and Group III by Mann-Whitney U test.

DISCUSSION

Avulsions are the worst of dentoalveolar injuries because it causes complete separation of tooth from the alveolar socket, tearing of the PDL and leaving viable PDL cells on root surface [9]. The PDL

cells which partly comprises of periodontal ligament fibroblasts are responsible for the reattachment of the avulsed tooth following reimplantation into its socket [9]. In majority of situations replantation is the treatment of choice but cannot always be immediately carried out [10].

Hammer in 1955 explained that the periodontium reacts to replantation in different ways, of which the favourable outcomes include healing with normal PDLor with surface resorption [11]. Since these reactions occur in the PDL space, treatment outcome entirely depends on the vitality of the cells of periodontium [11,12]. In situations where replantation is delayed, the tooth can be stored in suitable storage media to avoid dehydration and to maintain the tooth vitality [9]. Replantation is not indicated in teeth with enamel defects, dental caries, restorations and periodontal disease, hence these were eliminated from this study. Fibroblast function is known to be affected by age, trauma and inflammation. Therefore, the teeth selected in this study were non-carious mature human premolar teeth without any periodontal disease, undergoing extraction for orthodontic therapeutic purposes from young healthy individuals [5].

Among the factors to be considered prior to reimplantation, the potential of the storage medium in maintaining the vitality of cells is considered critical than the extra-alveolar time, in preventing unfavourable root resorption [10,12].

A critical duration at which even though harm has occurred to many PDL cells, remaining cells being attainable for estimation appeared to be 30 minutes, hence 30-minutes dry time was selected for this study. Thirty minutes also represented an actual situation in which any tooth after its avulsion may be left dry before its transfer into a transport medium. Avulsed tooth replantation within 30 minutes presented a higher success rate [3,7,13,14]. In the present study, the time duration selected for storage of avulsed teeth in the experimental media was 45 minutes as it helped in relating with former studies which used similar methodology [6-8,15].

The efficacy of any storage media is dependent on the type of media, its characteristics and the amount time out of the socket. An ideal storage medium should be suitable in maintaining the viability, clonogenic capacity, mitogenicity of an injured PDL, have a physiological osmolality (230 to 400 mOsmol/kg), pH (6.6-7.8) and when maintained at a suitable temperature, would allow maximum cell growth or its survival [3,16]. Such a medium is rarely possible to obtain. Hence, an ideal storage medium can be considered as one that shows maximal PDL cell viability and which is easily obtainable at the time of emergency [12,17]. Moist media was considered better than a dry storage medium. Water, saliva, and saline were used as a medium but was proved to be ineffective due to bacterial contamination or the hypotonic effect that lead to cell death [18]. In the present study, commercially available HBSS was compared with naturally available products like milk and aloe vera as a storage medium.

Hank's Balanced Salt Solution (HBSS) used in biomedical research is considered to be non-toxic, pH balanced, has an osmolarity of 270-290 mOsm/L and contains many essential nutrients. It is available as "Save-A-Tooth" in pharmacies [19].

Trope in 1995 suggested that when an avulsed tooth is soaked in HBSS for about 30 minutes following a 60 minutes extra oral dry time, the chances of success after reimplantation, was high. Due to its varied properties like preservation and renewal of degenerated PDL cells of avulsed teeth, it has been recommended by the American Association of Endodontics in 1995 to be the ideal storage medium [2,20]. Hence in the present study, HBSS has been used as one of the experimental storage media.

HBSS has not been recommended to the public due to its high cost and the lack of availability at the site of an accident [3,21].

Milk, which is the most convenient, cheapest, and readily available solution in most situations, was recommended as the best alternative by The American Association of Endodontists in 1994 [22,23]. Milk was used in this study because of its specific characteristics, such as low bacterial content, absence of active toxicants that could be detrimental to PDL cell viability [24], physiological osmolality (275 milliosmol/kg), neutral pH (6.5-6.8) and the presence of nutrients and growth factors that are supplied to the PDLF cells [8,19]. Bovine milk contains Casein Phosphopeptides (CPP) which functions as carriers for different minerals [23]. Milk also contains epidermal growth factor which was found to fasten osteoclasia, decreasing possibility of ankylosis and the likeliness of replacement resorption [24].

On comparing the various presentations of milk by Marino T et al., regular pasteurised milk and long shelf-life Ultra High Temperature (UTH) pasteurised whole milk showed no significant difference at any time period [1,25].

Milk has an additional advantage of reproducing PDL precursor cells in a chilled state as reported in previous studies by Blomlof L and Otteskog P, and Lekic PC et al., [22,26].

According to studies by Harbacz OM et al., Souza BD et al., Souza BD et al., and Saluja KS and Anegundi RT, skimmed milk had better performance than milk with a higher fat content [27-30]. Though studies showed that whole milk performed better than skimmed milk at 24 hours, previous studies proved both skimmed milk and whole milk were effective in retaining the viability of PDLF up to 48 hours [21,29,31]. Hence, the milk was used in the present study was regular pasteurized low fat milk in a chilled state. Previous studies recommended that preservation of the avulsed tooth in milk or HBSS, as they proved to be superior to other solutions [15,32-34].

Few studies which compared different kinds of milk with HBSS found that milk had better performance than HBSS in preserving PDL cell vitality for more than 24-hours [18,24,29,35]. Contrasting results were found in previous studies, which had reported better effectiveness of HBSS in relation to milk [4,17,19,36].

In the present study, though HBSS had greater number of viable cells (921.40±608.438) than milk (812.70±449.170), the difference was not statistically significant ($p>0.05$). The results were similar to studies which places milk equal to HBSS as a storage medium [6,8,21,22].

Milk contains important nutrients, such as carbohydrates, amino acids, and vitamins, which is probably the reason why these results were favourable. Also, in milk available commercially, the enzymes detrimental to the PDLF may be inactivated by pasteurisation [3].

Consequent to the increasing interest in usage of naturally available products for avulsed teeth, Aloe Vera (Barbadensis), a commonly available natural plant, was also tested. It has anti-bacterial, anti-inflammatory, antioxidant, immune boosting, and hypoglycemic properties along with an optimal pH 6 [2,9]. The osmolarity of aloe vera was found to range from 280-300 mOsm/L which was optimal for cell growth. Fresh aloe vera extract was used in this study as the chances of availability could be relatively high.

In the present study, the number of viable cells in Aloe vera (241.00±194.572) was significantly lower ($p<0.05$) when compared with HBSS (921.40±608.438) and milk (812.70±449.170), which was in accordance with many other studies [2,9,37].

Aloe vera exhibited highest viable cells when compared to egg white and milk, which was in contrast to the present result [5]. Another study assessed aloe vera as a storage medium in maintaining the cell viability of dry-stored dog teeth and found comparable results with aloe vera at concentration of 50% and HBSS, but they were superior to milk [38].

Another point to note was that in the present study, authors used 100% aloe vera extract which showed lower ability to preserve vital cells. Aloe vera at concentration of 10%, 30%, 50% performed similarly and had the greatest capacity in maintaining the cell viability when compared with aloe vera at concentration of 100% which showed smallest ability in preserving the PDL cells viability. The mean pH level of the 100% concentration of aloe vera is more acidic (mean pH level=5.21) compared with other experimented concentrations of aloe vera extract. This would be the possible explanation why aloe vera showed lower efficacy in the present study compared to HBSS and milk [14].

Another probable reason which may have reduced its effectiveness in preserving cell viability might be the fact that, since pure aloe vera is highly viscous, at the time of experiment it entirely covered the surface of the experimented cells and may have possibly prevented the accessibility of oxygen to the cells. Thus, increased cell death occured due to decrease in the availability of oxygen [14].

Techniques tested for estimation of the number of viable PDL cells in an avulsed tooth are numerous [39]. In the current study, the root surfaces were treated with collagenase and dispase, to reduce exposure of active trypsin on the cells and to retain maximal cell viability, as was executed by various authors [6,7,39].

This procedure allowed rapid cell retrieval and maintained maximum cellular integrity [39]. Collagenase and dispase enzymes cause the efflux of cells by disruption of the extracellular matrix but without enormous destruction of their own membrane. Also, this method closely approximated the typical clinical scenarios the cells do not undergo long processing times [15]. Trypan blue dye exclusion assay was found to be the most sensitive assay to differentiate viable cells from nonviable ones [40]. This method is cheap and can be easily performed. When viewed under a light microscope, the viable cells will have a clear cytoplasm but non viable cells will have a blue cytoplasm. In the present study, a single observer carried out the counting of viable cells. This reduced variability in quantification process. Subjectiveness in determination of cell numbers, time consumption, intensive labor adds to the drawbacks of this method [17].

LIMITATION

Limitations and variability exists in the present study design, as with any other *in vitro* study. Previous studies suggested the usage of extracted teeth for simulation of avulsed teeth. In this investigation, though it was a single operator who carried out the extractions, the force exerted for individual extractions would have been different, which could have produced variable trauma during extraction, translating variability in periodontal cell vitality counts.

CONCLUSION

Within the limitations of this study, it was found that the efficacy of HBSS and regular pasteurized low fat milk in a chilled state was comparable but superior to aloe vera extract at the concentration of 100%. The knowledge obtained through this study could be made a part of public awareness programs, in order to properly handle and save a large number of avulsed teeth, the prognosis of which can gently improve if placed in an appropriate storage medium.

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