ORIGINAL RESEARCH

Comparison of Hank's balanced salt solution, Low fat milk, Soymilk and Aloevera extract on periodontal ligament cell viability

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ABSTRACT

Objectives: The purpose of this study was to evaluate and compare the ability of Hank's balanced salt solution (HBSS), Low fat milk, Soymilk, and Aloevera extract to maintain human periodontal ligament (PDL) cell viability *in vitro*. **Method:** PDL cells were obtained from extracted healthy premolars and cultured in Dulbecco's modified Eagle's medium (DMEM). The cultures were exposed for 1, 3, 6, 9 and 24 hours to experimental solutions (DMEM was positive control). Cells in the different media were examined under the optical microscope and their viabilities were analyzed by MTT assay. **Results:** The data were statistically analysed using Kruskal Wallis ANOVA and Mann Whitney 'U' test. Statistical analysis showed that the efficacy of Soymilk on PDL cell viability at 3hours was significantly higher than HBSS, low fat milk, Aloevera extract and control Group (DMEM) ($p<0.05$). Performance of low fat milk and Soy milk was equal to control group (DMEM) at 6, 9 and 24 hours. HBSS and Aloevera maintained significantly less viable PDL cells compared to Low fat milk and Soy milk. **Conclusion:** The results indicate that Soy milk and Low fat milk can be used as a suitable medium for avulsed tooth up to 24 hours.

Keywords: PDL; Fibroblast; HBSS; Low fat milk; Soy milk

INTRODUCTION

World Health Organization has defined avulsion as the complete displacement of a tooth from its alveolar socket due to traumatic injury[1]. Tooth avulsion is one of the most severe dental traumas. Its prevalence is approximately 1-16% of all traumatic injuries to the permanent dentition[2]. Andreasen and Andreasen (1990) predicted that the incidence of these injuries might surpass the incidence of dental caries[3]. The causative factors are trauma after fighting, falls, sports and bumps against hard objects or the floor[4].

Avulsion causes rupture of neurovascular bundle which leads to loss of pulp vitality[5]and necrosis of periodontal ligament. Necrosed periodontal ligament (PDL) cells in replanted tooth promote inflammatory process and in severe situations leads to replacement, root resorption and loss of tooth[6]. Ideal treatment is immediate replantation for maintaining the viability of periodontal ligament (PDL) cells. However, it is not possible, because of factors like the person's conscious state, lack of first aid knowledge and confidence in people gathered at the scene of accident. Initially it was thought that success of replantation is associated with the speed with which the tooth is

replanted but now researchers have demonstrated that storage medium is one of the most important factors than the extra oral time. A storage medium may be defined as a physiological solution that closely replicates the oral environment to help preserve the viability of PDL cells following avulsion[7]. Various synthetic and natural storage medias were studied which include HBSS, Viaspan, Minimum essential medium, contact lens solution, normal saline, milk, propolis, coconut water, Green tea, egg white, saline and so on[8]. An ideal storage medium should provide physiological pH, osmolarity, it should have antibiotic and anti-inflammatory properties clonogenic and mitogenic properties, should be easily available at the site of accident and should be economical[9]. Unfortunately, such an ideal storage medium has not been discovered yet.

Hank's balanced salt solution (HBSS) is recommended by AAE as storage media of choice[10]. It has appropriate pH and osmolality[11], non-toxic and contains many essential nutrients[12]. However it is not available at the site of trauma, so several investigations have been carried out to identify the suitable alternative media. Milk contains nutrients such as carbohydrates, vitamins and growth factors. It has adequate pH and osmolarity[13]. Bacterial content of regular pasteurized milk is low compared to bovine milk due to pasteurization process[14]. However milk has short life and needs refrigeration, researchers started examining the long shelf-life milk to overcome these disadvantages. Soy milk is a long shelf- life milk. It is rich in many essential amino acids, little saturated fat and no cholesterol. But potential use of Soy milk for tooth storage has been poorly evaluated[15].

Similarly Aloevera has recently gained popularity in the medicinal field because of its antidiabetic, anticancer, antibiotic and anti- inflammatory properties[16]. To the best of our knowledge, literature is scarce about the use of aloevera extract as a storage media for avulsed teeth.

There are numerous studies on HBSS and milk but very few studies on Soy milk and Aloe Vera. So the present study was carried out to check and compare the PDL cell Viability of HBSS, Low fat Milk, Soy Milk and Aloevera extract.

MATERIALS AND METHODS

The study was conducted at the Department of Pedodontics and Preventive Dentistry, College of Dental Sciences, Davangere and the Department of Oral and Maxillofacial Pathology and Microbiology, Maratha Mandal's Nathajirao G. Halgekar Institute of Dental Sciences and Research centre Belgaum-590010. This study was approved by institutional review board, College of Dental sciences, Davangere. Written consent was obtained from the patient.

Cell Culture of Human PDL Cells: PDL cells were collected from healthy premolars extracted for orthodontic purposes. Extraction was performed atraumatically and then tooth was washed in sterile saline solution to wash out residual blood. The tooth was stored in a conical tube filled with HBSS. The tooth was held with forceps at the coronal region, and the PDL cells were obtained by scrapping with a no. 11scalpel blade from the middle third of the root surfaces. The tissues were split into small pieces and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37^0C. The PDL cells outgrown from PDL tissues expressed a fibroblast-likephenotype and were allowed to reach confluence and passed at a 1:2 ratio until they were used for the experiment.

Experimental Groups: Experimental PDL cells were washed by phosphate-buffered saline, and these cells were exposed to different experimental solutions. The storage solutions used in the experiments were as follows: (1) Group I: HBSS, (2) Group II: low fat milk, (3) Group III: Soymilk (4) Group IV: Aloevera extract.

ASSESSING CELL VIABILITY

By3-(4,5-dimethylthiazol-2-yl)-5(3 arboxymethonyphenol)-2-(4-sulfophenol)-2-(4-sulphophenyl)-2H-tetrazolium (MTT) assay.

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay. For each experiment, 5 x 10^3 cells in supplemented DMEM was plated in 96-well tissue- culture plates and incubated at37^0C in5% CO2 and 95% air for 24 hours. Subsequently, the medium was removed and 200µl of each of the different experimental solutions were added. The plates (n =3) were maintained at 37 ^{0}C in 5% CO2 for 1,3,6,9 and 24 hours. After the appropriate time, the MTT solution (20 µl/ml) was placed in each well, and the plates were incubated at 37 ^{0}C in 5% CO2 and 95% air for additional 4 hours. To quantify the viability of metabolically active cells, the optical density (OD) of the solubalized formazan product was measured by means of spectrophotometer at a 490 nm wavelength[17].

Statistical Analysis: Comparison of 5 groups at different time intervals was done by Kruskal Wallis ANOVA (Table 1). Pair wise comparison of all experimental groups with respect to OD was done by Mann-Whitney U test (Table 2).

RESULTS

Kruskal Wallis ANOVA test (Table 1) showed statistically significant difference among all the groups at 3, 6, 9 and 24 hours interval with p values 0.0170, 0.0180, 0.0320, 0.0240 respectively except at 1hr (p=0.1090). Soymilk showed significantly higher number of PDL cells (201%) at 3h (Table 1) compared to all other tested media. Even

at 24h (Table 2) mean percentage of PDL cells in Soymilk was high followed by low fat milk, HBSS, and Aloevera extract showed the least percentage. When pair wise comparison was done by Mann-Whitney U test (Table 2) at 1hr, there was no statistically significant difference observed in all the test groups except control group which showed statistically significant difference with Group I (HBSS), Group II (low fat milk) and Group III (Soy milk), (p value was 0.0495) although there was no statistically significant difference found between control group and Group IV (aloevera extract), (p=0.2752).(Table 2) efficacy of Soy milk on PDL cell viability was significantly higher than other groups (p<0.05)at 3hr. However performance of low fat milk and Soy milk was equal to control group (DMEM) at 6, 9 and 24 hours. HBSS and Aloevera showed significantly less viable PDL cells compared to Low fat milk and Soy milk at all tested time intervals except at 1hr.

Table 1: Comparison of five study groups (I, II, III, IV and control) with respect to efficacy at 1hr, 3hrs, 6 hrs, 8hrs and 24 hrs by Kruskal Wallis ANOVA

Time points	Groups	Mean	SD	SE	Mean rank	Percentage Of PDL viable cells	H-value	P-value
1 hour	Group I	0.455	0.009	0.005	10.33	113%	7.5690	0.1090
	Group II	0.450	0.005	0.003	8.83	112%		
	Group III	0.473	0.042	0.024	11.67	118%		
	Group IV	0.427	0.032	0.018	6.50	106%		
	Control	0.400	0.029	0.017	2.67	100%		
3 hours	Group I	0.430	0.014	0.008	6.00	112%	12.0330	0.0170*
	Group II	0.519	0.037	0.022	11.00	135%		
	Group III	0.773	0.188	0.109	14.00	201%		
	Group IV	0.429	0.050	0.029	6.33	111%		
	Control	0.384	0.027	0.015	2.67	100%		
6 hours	Group I	0.350	0.002	0.001	4.00	97%	11.9010	0.0180*
	Group II	0.384	0.004	0.002	13.17	106%		
	Group III	0.380	0.004	0.002	11.33	105%		
	Group IV	0.352	0.008	0.005	3.00	97%		
	Control	0.373	0.006	0.003	8.50	100%		
9 hours	Group I	0.347	0.007	0.004	4.33	94%	10.5860	0.0320*
	Group II	0.369	0.008	0.004	11.50	100%		
	Group III	0.369	0.003	0.002	11.33	100%		
	Group IV	0.338	0.013	0.008	2.67	92%		
	Control	0.366	0.005	0.003	10.17	100%		
24 hours	Group I	0.251	0.008	0.004	5.00	82%	11.2280	0.0240*
	Group II	0.290	0.005	0.003	9.67	95%		
	Group III	0.302	0.029	0.017	11.83	99%		
	Group IV	0.225	0.022	0.013	2.00	74%		
	Control	0.304	0.013	0.007	11.50	100%		

*p<0.05

Table 2: Pair wise comparison of five study groups (I, II, III, IV and control) with respect to efficacy at 1hr, 3hrs, 6 hrs, 8hrs and 24 hrs by Mann-Whitney U test

Time points	Groups	Group I	Group II	Group III	Group IV	Control
1 hour	Mean	0.455	0.450	0.473	0.427	0.400
	SD	0.009	0.005	0.042	0.032	0.029
	Group I	-				
	Group II	p=0.3827	-			
	Group III	p=0.5127	p=0.5127	-		
	Group IV	p=0.3827	p=0.5127	p=0.1266	-	
	Control	p=0.0495*	p=0.0495*	p=0.0495*	p=0.2752	-
	Mean	0.430	0.519	0.773	0.429	0.384
	SD	0.014	0.037	0.188	0.050	0.027
	Group I	-				
	Group II	p=0.0495*				
	Group III	p=0.0495*	p=0.0495*			

3 hours	Group IV	p=0.5127	p=0.0495*	p=0.0495*		
	Control	p=0.0495*	p=0.0495*	p=0.0495*	p=0.2752	-
6 hours	Mean	0.350	0.384	0.380	0.352	0.373
	SD	0.002	0.004	0.004	0.008	0.006
	Group I	-				
	Group II	p=0.0495*	-			
	Group III	p=0.0495*	p=0.2752	-		
	Group IV	p=0.5127	p=0.0495*	p=0.0495*	-	
	Control	p=0.0495*	p=0.0809	p=0.1266	p=0.0495*	-
9 hours	Mean	0.347	0.369	0.369	0.338	0.366
	SD	0.007	0.008	0.003	0.013	0.005
	Group I	-				
	Group II	p=0.0495*	-			
	Group III	p=0.0495*	p=0.8273	-		
	Group IV	p=0.2752	p=0.0495*	p=0.0495*	-	
	Control	p=0.0495*	p=0.6625	p=0.5127	p=0.0495*	-
24 hours	Mean	0.251	0.290	0.302	0.225	0.304
	SD	0.008	0.005	0.029	0.022	0.013
	Group I	-				
	Group II	p=0.0495*				
	Group III	p=0.0495*	p=0.5127			
	Group IV	p=0.0495*	p=0.0495*	p=0.0495*		
	Control	p=0.0495*	p=0.2752	p=0.6625	p=0.0495*	-

*p<0.05

DISCUSSION

Tooth avulsion is one of the most severe among dental traumas. It is more common in a newly erupted teeth and its prevalence is approximately 1- 16% of all traumatic injuries to the permanent dentition[2]. Avulsion causes rupture of neurovascular bundle which leads to the loss of pulp vitality[5] and necrosis of periodontal ligament. Necrosed periodontal ligament (PDL) cells in replanted tooth promote inflammatory process and in severe situations lead to replacement root resorption and loss of tooth[6]. The primary goal is to preserve the viability of the PDL cells attached to the root surface until appropriate treatment can be performed. Extraoral time and storage medium are the critical factors responsible for prognosis of avulsed tooth. Ideally, the tooth should be replanted immediately to minimize the incidence of root resorption and ankylosis. However because of the limitations to replant the exarticulated tooth at the accident site immediately, it is important that the exarticulated tooth to be transported in a good storage medium to the dentist to achieve favourable prognosis[7]. An ideal storage medium should provide physiological pH, osmolarity, should have antibiotic and anti-inflammatory properties, clonogenic and mitogenic properties, should be easily available at the site of accident and economical[9]. Unfortunately, such an ideal storage medium has not been discovered yet. According to American Academy of Endodontics (AAE), Hank's balanced salt solution (HBSS) is storage media of choice[10]. Disadvantage of HBSS is that, it is not available to the public easily, hence an alternative medium which is readily available and inexpensive is required. The present study was aimed to assess novel transport mediums which are economical, easily available and helps in preserving the viability of PDL cells at various time intervals. In this study, transport mediums studied were HBSS, low fat milk, Soy milk and Aloevera extract with Dulbecco's modified eagles medium (DMEM) which was taken as control because it contains approximately four times as much of the vitamins and amino acids present than Eagle's modified essential medium (EMEM) formulation and 2–4 times as much glucose. In addition, it contains iron and phenol red. DMEM is suitable for most types of cells[18], hence it was chosen in the present study. HBSS is nontoxic, standard saline solution that is widely used for biomedical research[19]. Due to properties of HBSS like preservation and renewal of the degenerated periodontal ligament cells of avulsed teeth and maintenance of superior success rate, it has been recommended by the American Association of Endodontics as the ideal storage medium[20, 21] but its widespread use is doubtful, because it may not be readily available in many occasions in which tooth avulsions are likely to occur. Milk has been studied extensively [14,22, 23, 24,] and has gained wide acceptance as a suitable medium for avulsed teeth. Soy milk is a beverage made from soybeans. Physiologic osmolality is 267mOsml/kg[25] and has many nutrients for maintaining the viability of PDL cells. So we chose Soy milk as a storage medium for the study.In the present study, aloevera extract was chosen as one of the transport medium because of its physiologic osmolarity (280-300mOsm/l)[26]. Eight essential amino acids and 11 of 14 secondary amino acids are found in aloe vera. It is thought to promote healing; therefore it

can be used in surgical wounds, in root canal treatment as an analgesic dressing, and around dental implants to control inflammation. In a recent study, human kidney cell death rate was found to be reduced by two-thirds when cultured in aloevera gel[16].

About the method used to assess cell viability, trypan blue staining have shown less sensitivity because it does not characterize the metabolic condition of the cell or its actual physiological health[27]. In this way, an MTT assay should be a more sensitive tool for cell viability analysis than trypan blue staining [13]. Hence we used MTT assay for counting the number of the cells. This assay measures the viable cells which are metabolically active and is based on using a yellow tetrazolium salt which changes to insoluble formazan crystal in purple hue. These changes are only accomplished in metabolically active cells by the action of dehydrogenase enzymes. Therefore the more brown hue indicates the more viable cells presence. Compared to other methods, this assay is more reliable, efficient and faster. On the other hand, the Elisa reader device could eliminate intra- and interexaminer variations[28]At 1hr, all of the tested storage media had similar abilities to maintain cell viability. This similarity might be attributed to the use of the cell's own homeostasis system to maintain the normal physiology[22]. During this period, the nutrients present in the storage media may not be required for the maintenance of cell viability. After 2h in extraoral conditions, cells deplete their stored metabolites, requiring a supply of essential nutrients to preserve viability[5].In the present study, results have demonstrated that Group I (HBSS) and Group II (Aloevera extract) performed equally in maintaining the PDL cell viability till 9hr with no statistically significant difference ,although HBSS showed higher percentage at 1hr (113%) compared to Aloevera which showed 106%. At 3, 6 and 9hrs Aloevera showed comparable results to HBSS. These findings are in accordance with previous study by Punit Fulzule et al[26] who studied the viabibilty of PDL cells in HBSS and Aloevera at 15,30,60,90 and 120min and stated that Aloevera gel exhibited PDL cell viability comparable to HBSS. However at 24hr HBSS maintained significantly higher number of PDL cells compared to Aloevera extract.

HBSS significantly maintained less number of PDL cells compared to low fat milk, Soy milk and DMEM till 24hr except at 1hr. This interpretation is in accordance with the studies by Wen-jun Wang et al[29], B.D.M Souza et al[13] and Marino et al[14] who obtained better results with milk when compared to HBSS. This contradicts the findings of other authors who showed that HBSS was better than milk after 12 h, Hiltz & Trope[12] or during all experimental period Huang et al[30] and recently study conducted by Priya Rajendran et al[31] also showed better results with HBSS. The reason for the differing results might be attributed to the type of milk and storage time of HBSS. Low fat milk maintained PDL cells equal to Soy milk and the control Group (DMEM) except at 3hrs where Soy milk displayed higher number PDL cells. These findings are similar to the recent study by Emmanuel et al[25] who evaluated and compared the efficacy of Soy milk with DMEM, whole milk, HBSS coconut water, egg white and Gatorade at 2, 12 and 24h. Statistical analysis showed that DMEM, whole milk, and Soymilk were the most effective media for maintaining cell viability at all tested times ($p < 0.05$). But low fat milk significantly maintained higher number of PDL cells compared to HBSS till 24hrs. This finding is in agreement with those of Sigalas et al[24] and Ashkenazi et al[23] who affirmed that cold milk is suitable for the preservation and proliferation capacity of PDLF. Recent study conducted by Karanveer Singh Saluja and Rajesh T. Anegundi[32]showed that low fat milk preserved significantly more viable PDL cells compared to Medium and High fat milk. However, some authors have concluded that milk is effective for only a short period of time[30]. It is likely that methodological differences like the storage temperature and the type of milk used can explain this variation. In the present study Low fat milk also maintained significantly higher number of PDL cells till 24 hrs when compared to Aloevera extract. In contrast to this, study conducted by Meenakshi Sharma et al[16], showed that Aloevera significantly maintained higher number of PDL cells compared to milk. The reason could be attributed to the methodology where they used tryphan blue staining which is less sensitive technique compared to MTT assay. One possible explanation for the positive results obtained with milk is that, besides the physiological pH, osmolarity and the presence of cytoprotective effect of nutrients, it also contains growth factors. Recent studies showed that bovine milk contains transforming growth factor beta (TGF-b), platelet-derived growth factor (PDGF),insulin-like growth factor 1 (IGF-1), and fibroblast growth factor (FGF), which are resistant to pasteurization. These growth factors aids in proliferation and differentiation of periodontal ligament cells and hence periodontal regeneration[15]. HBSS storage may cause alterations in the concentrations of its components, making this solution unable to adequately nourish the cells for more than 6hrs. Recently, Souza et al [13] concluded that the storage time of HBSS has a negative influence on its ability to maintain PDLF viability. HBSS in our study was refrigerated and stored for more than 3 months. This could have predisposed to HBSS displaying inferior results.At 3hrs, Soy milk significantly maintained highest number of PDL cells compared to all the groups which indicates Soy milk is a superior storage medium till 3hrs. The improved viability of cells stored in Soy milk may be due to the cytoprotective effects of nutrient constituents such as carbohydrates, sugars, proteins, fats, calcium, vitamin A, C, D and E, as well as the physiological pH. Such components may help nourishing the cells and maintaining their viability, which makes Soy milk an

alternative to other milk formulations as a storage medium[33].Soy milk showed significantly higher number viable cells compared to HBSS at all tested times except at 1hr. These results are similar to the findings of Moura et al[34]who showed higher viability with Soy milk than HBSS. Improved viability of Soy milk could be due to its nutrients. However equal efficacy was obtained with Soy milk and HBSS as study conducted by Emmanuel et al[25, 35]and Fariborz Mozami et al[15]. Soy milk performed equal to low fat milk till 24 hrs except at 3hrs where it significantly maintained higher viability of PDL cells comparable to low fat milk. Similar results were obtained with Emmanuel J. N. L. Silva et al[25] who showed that the efficacy of Soy milk in maintaining the viability of 3T3 fibroblasts is similar to that of HBSS and milk. In contrast to the present study, Camilla Christian Gomes Moura et al[33] showed that milk was less effective in preserving the PDL cells compared to Soy milk. Difference in the result could be due to the type of milk used.Except at 1hr Soy milk maintained significantly more number of viable PDL cells compared Aloevera extract. To the best of our knowledge comparison of Soy milk and Aloevera have not been done in the previous studies.In the present study, performance of Soy milk was similar to control Group (DMEM).This was in accordance with the study conducted by other authors [33,15,25].Aloevera performed equal to HBSS in maintaining the PDL cell viability till 9hrs. This finding is similar to the study conducted by Punit Fulzele et al[26] where they showed that efficacy of Aloevera exract and HBSS are similar but number of viable cells was significantly less in Aloevera at 24 hrs when compared to HBSS. In the present study when compared to Lowfat milk and Soy milk, Aloevera maintained significantly less number of PDL cells till 24hs except at 1hr. Studies have shown that, in cool conditions, cells have a higher percentage of viability than at room temperature, as cooler temperatures reduce cell swelling, increase cell viability, and improve recovery, all of which promote wound healing[22,11]. The reason for under performance of aloevera could be due to the room temperature used. In our study HBSS, lowfat milk and Soy milk were stored at 3^0 C where as Aloevera extract was freshly prepared and used at room temperature because this plant can be found commonly in most households, nurseries, parks, recreation areas and is available at the site of trauma. However it performed equal to DMEM at 1hr and 3hr. These findings are similar to the Study conducted by Samaneh Badakhsh et al[25].

Even though there was statistically insignificant results seen in between the groups at 24hrs, percentage wise when experimental groups were compared at 24hr (Table 1), Soy milk showed the highest percentage 99%, followed by low fat milk 95%, HBSS 82% and Aloevera 74%. However DMEM was maintaining its efficacy at all tested time intervals. This shows that the viable PDL cells decline as the time of storage increases. Present study design presents some limitations. The number of cells in each experimental group was not standardized. The in vitro culture of PDL cells do not simulate the oral environment. However there is lack of adequate research in evaluating efficacy of Soy milk and Aloevera extract as a storage media for avulsed tooth. Further, more number of research are required to evaluate the effectiveness of Soy milk and Aloevera extract. Hence we recommend future research on these storage medias before considering their usage clinically.

This study concludes that

1. Soymilk demonstrated highest number of viable cells at 3hr
2. Low fat milk and Soy milk are equal to DMEM at 6, 9 and 24hrs
3. HBSS and Aloevera were not effective in preserving the PDL cells as good as low fat milk and Soy milk.

Hence with the present research we could recommend to use low fat milk and Soy milk as a storage medium for avulsed tooth at the site of trauma during avulsion.

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