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Viability of periodontal ligament cells in selected transport media for avulsed teeth: an *in vitro* study

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Aim: To compare the viability of periodontal ligament (PDL) cells stored in Hanks Balanced Salt Solution (HBSS) with those in readily available transport media over a variable period of time. **Methods:** Periodontal ligament cells harvested from premolars freshly extracted for orthodontic reasons were cultured for exponential growth. The cells were exposed to egg white, evaporated milk, water and Hanks Balanced Salt Solution (HBSS) at room temperature. Their viability was evaluated after 30 minutes, 1 hour and 3 hours with the tetrazolium salt-based colorimetric assay (MTT assay). Statistical analysis was done using the IBM® SPSS version 23.0 software. Comparison between the Mean Optical Densities (MODs) of the cells stored in HBSS and other media at each time interval was done using the independent t test. Repeated measure ANOVA and Tukey post-hoc test were also carried out to compare the MOD of cells within each medium over time. The level of significance was set at $p < 0.05$. **Result:** The PDL cells stored in egg white had higher MODs than those in HBSS at 30 minutes and 1 hour. Conversely, the MODs of the cells stored in milk and water were lower than those in HBSS at all the studied points. There was a significant difference in the viability of the cells stored in HBSS and water at all the time points ($p<0.05$). **Conclusion:** For up to an hour, egg white was found to perform better than HBSS in supporting the viability of PDL cells.

Keywords: Periodontal ligament. Hanks Balanced Salt Solution. Tooth avulsion. Egg white. Milk.

Introduction

Dental avulsion is considered one of the few emergencies in dentistry. If not well managed, it can result in permanent loss of the affected tooth, which could necessitate an artificial replacement. In Nigeria, recent studies reported prevalence rates of 2.5% and 10.4% for dental avulsion in school children[1,2]. These values fall within a documented worldwide prevalence rate of 0.5-16%. Although its prevalence is considered low, dental avulsion has been reported to have an impact on the quality of life of the few affected individuals[3]. Immediate replantation of an avulsed tooth is highly advocated, as the prognosis of the tooth depends on the viability of the periodontal ligament (PDL) cells at the time of replantation. However, immediate replantation may not be practicable, hence the need for transport media that can preserve the PDL cells until replantation[4]. Such media need to be readily accessible at sites of injury.

Hanks Balanced Salt Solution (HBSS) has been shown to be effective in supporting the mitogenic and clonogenic capacity of PDL cells for up to 48 hours[5-8]. Hence, it is recommended as one of the preferred transport media for avulsed teeth by the International Association of Dental Traumatology (IADT)[4,9]. HBSS is mainly used in laboratories; in order to make it readily available, a kit system which suspends the avulsed tooth in a basket placed in HBSS was manufactured (Save a tooth; Phoenix Lazerus. Inc., Pottstown, PA,USA)[8,10]. However, HBSS is expensive and not readily available in developing countries, thereby limiting its use. In addition, some studies have documented that other media, such as milk, supported the viability of PDL cells better than HBSS[11,12]. Milk is an osmolality balanced medium that has been recommended as a transport medium for avulsed teeth and preferred to HBSS owing to its availability[4,8]. It contains amino acids and vitamins which can help in nurturing the PDL cells. It also contains epithelial growth factors (EGF) which initiates the proliferation of epithelial rest cells of malassez and activates resorption of the alveolar bone[13]. This resorptive ability of milk reduces the likelihood of ankylosis. Furthermore, its pH and osmolality fall within the recommended range of 6.5 to 7.2 and 270mosmol/kg, respectively[8]. Despite its being recommended, the maximum period of its use in maintaining the viability of PDL cells is still controversial, with most reports varying between 2 and 6 hours[14,15]. Milk exists in different forms, with varying fat content. Low fat milk has been reported to be more effective than the full cream milk in preserving the viability of PDL cells[16]. Also, comparison of the viability of PDL cells in milk with those stored in HBSS has yielded conflicting results, which may be due to the differences in the composition of the milk used and differences in methodology[11,12,17-19].

Numerous natural substances, such as propolis, rice water, aloe vera juice, green tea extract and coconut water, have also been investigated for their possible use as media for transporting avulsed teeth[11,18,20,21]. In Nigeria, the egg white of chicken is a relatively common, cheap and readily accessible option. It has been shown in other environments to be effective for up to about 10 hours in preserving the viability of PDL cells as a result of its near physiologic pH, osmolality and absence of microbial contamination[22]. Similarly, it contains dextrose, nitrogen, sodium chloride and proteins, which

are highly nutritive[20]. Khademi et al.[23] (2008), in their *in vitro* study, reported that cells stored in egg white had similar viability for up to 6-10 hours with those in HBSS; variations may, however, exist in egg composition with respect to the breed and feed of the chicken. On the contrary, though water is a readily available and affordable medium, it has been shown to be a poor medium for the transport of avulsed teeth. This is as a result of its hypo-osmolarity and lack of nutrients[11,16,24]. The IADT[4] only recommends the use of water in the absence of other media to prevent the dehydration of the avulsed tooth. Differences in composition exists with the source of eggs and milk[25]. In Nigeria, there is a paucity of data on the effectiveness of these media in supporting the viability of PDL cells over time. This study, therefore, determined the viability of Periodontal Ligament cells in evaporated milk, egg white and water in comparison to their viability in Hanks Balanced Salt Solution.

Materials and Methods

Ethical approval for the study was obtained from the University of Ibadan/University College Hospital Ethics Review Committee (UI/EC/18/0665). This was an *in vitro* experimental study carried out at the Paediatric Dentistry Clinic of the Department of Child Oral Health, University College Hospital, Ibadan and the Tissue Culture Laboratory section of the World Health Organisation (WHO) National Polio Laboratory, Department of Virology, College of Medicine, University of Ibadan. Periodontal ligament cells were harvested from 8 premolars extracted for orthodontic purposes to simulate avulsion. In order to minimize contamination, the participants were made to rinse their mouth with 0.12% chlorhexidine mouth rinse (Corsodyl oral rinse USP, 0.12% Glaxosmithkline®, Puma Pharmaceuticals, Nottingham, UK) and the teeth to be extracted swabbed with nystatin (Kenstatin™ USP100,000 I.U/ml, Brussels Laboratories Pvt. Ltd, Ahmedabad (Gujarat), India) prior to the extraction. The extracted teeth were rinsed thrice in Phosphate Buffered Saline (PBS)(Sigma Aldrich®, Co, Spruce St., St. Loius) supplemented with 100IU/ml of penicillin, 100mg/ml of streptomycin (Gibco™, Life technologies) and 50µL/ml of nystatin, and subsequently transported to the laboratory in a 50 mL centrifuge tube containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco™, Life technologies).

Isolation and cell culture

All tissue manipulations were done in a Class II Biosafety Cabinet (BSC II), using standard laboratory procedures. Through the adoption of a modification of the technique by Somerman et al.[26] (1988), PDL fibres on the mid portion of the root were scraped with a number 15 surgical blade on a bard parker® blade handle into a petri dish containing DMEM. The tissue explant obtained was centrifuged at 3000 rpm for 1 minute. The supernatant was discarded, and 0.25% Trypsin EDTA was added to the tissue to break the intercellular bonds. After 30 seconds, trypsin action was halted with growth medium (DMEM supplemented with 10% foetal bovine serum (FBS). The cell suspension obtained was emptied into a T25 tissue culture flask, and this was incubated at 37°C for exponential growth. The first form of evidence of cell growth was seen within four weeks of culture, and the cells were about 80% confluent by the eighth week of culture. The cells that grew were elongated and

spindle-shaped, and they attained a whirled pattern at confluence, just as described in previous studies[18,27]. The cells were passaged into T 75 culture flasks for further exponential growth and elimination of other possible cell types. The cells from the 3rd -5th passage were used for the study.

Exposure of the periodontal ligament cells to the test media

The test media and their pH, which was determined using a pH indicator strip (ColorpHast®, EM Science, Associate of Merck, Germany), were as follows: egg white (eggs freshly obtained from the grocery) pH 8.5, evaporated milk (Three Crown™. FrieslandCampina WAMCO Nigeria PLC, Ikeja-Lagos Nigeria) pH 6.0, bottled water (Eva™, Nigerian Bottling Company, Lagos, Nigeria) pH 6.0, and HBSS (American BioInnovations™, Baltimore, USA) pH 7.0. The growth medium was decanted from all the flasks containing the PDL cells and the flasks were rinsed twice with PBS. Thereafter, 200 µL and 750 µL 0.25% trypsin-EDTA were added to the T25 and T75 flasks, respectively, for about one minute to detach the cells from the flask. The cell suspension obtained from the flasks was pooled together and the number of viable cells was determined using the trypan blue exclusion test. The cells were counted using an automated cell counter (Eve™, NanoEntek Inc. Korea) as 3.1 x 10^5 cells/ mL.

Afterwards, 100 µL of the cell suspension was seeded in each well of four tissue culture-treated 96-micro well plates (Corning Costar®) (a plate each for the baseline, 30 minutes, one hour and three hours incubation).

The plates were sealed and incubated at 37^0C for 48 hours to allow the cells to form a complete monolayer[24]. Following this, the growth medium was removed from each microwell and replaced with 100µL of each test medium based on the work design[17]. The cells were exposed to each of the test media for 30 minutes, one hour and three hours at room temperature (30°C). For each medium, 16 microwells were assigned for each test period.

Viability assay

The viability of the PDL cells was determined using the 3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. It was carried out as described in previous studies[17,28]. After each test period, the experimental storage media were removed, and the cells carefully washed 3 times with PBS to prevent any optical interaction of the remnant of the test media with MTT dye. For the coloured and viscous media, such as milk and egg white, during each rinse, the PBS was left for about 30 seconds to dilute the media before removal from the microwells. This was to ensure complete removal and prevent interaction with the MTT dye. Each well was filled with 25 µL of 5 mg/ml of MTT salt (Sigma Aldrich®, Co, Spruce St., St. Loius) dissolved in PBS and the plates incubated at 37°C. Following this, 125µL of DMSO (JHD®, Guangdong Guanghua Sci- Tech Co., Ltd, India) was added to the wells in order to dissolve the formazan crystals. Cell viability was determined by measuring the optical density (OD) at 490 nm on a spectrophotometer (Thermo LabSystems Multiskan RCMSEX® Thermo Electron Corporation® Finland) for each microwell.

Data Analysis

The OD values were exported to Microsoft Excel sheet for preliminary data analysis. Further analysis was performed using the Statistical Package for Social Sciences IBM (SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp). Test for normality was done using the Kolmogorov-Smirnov test and the data were found to be normally distributed. The Mean Optical Densities (MOD) and Standard Deviations (SD) of the cells in the microwells assigned for each medium at each time interval were calculated. The viability of the periodontal ligament cells stored in each medium at each interval was measured as the MOD of that medium at that time interval. Comparison between the MODs of the cells stored in HBSS and other media at each time interval was done using the independent t test. Repeated measure ANOVA with Tukey post-hoc test was also carried out to compare the MODs of the cells within each medium over time. Test of significance was set at $p< 0.05$.

Results

The baseline mean ±SD optical density for all media was 0.32±0.03. In the first hour, the cells stored in egg white had the highest MOD (0.27±0.03), followed by those in HBSS, evaporated milk and water. However, at 3 hours, similar MODs were obtained for cells in egg white and those in HBSS, as shown in Figure 1.

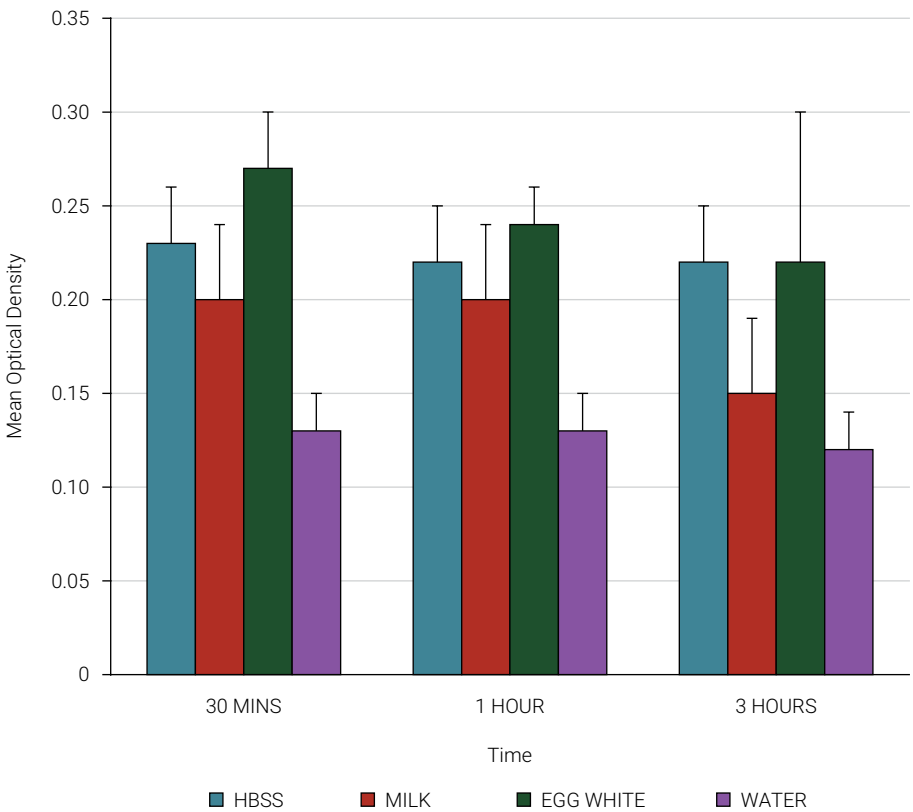

Figure 1. Distribution of the mean optical density of cells in the different media at the different time intervals

The MOD of the cells stored in water were lower than that for those in HBSS at all the time points ($p<0.05$), while the cells stored in egg white had a significantly higher MOD than those in HBSS at 30 minutes, as captured in Table 1

Table 1. Comparison of the mean optical density of PDL cells in HBSS and other media at the different time points

	HBSS	Milk	Egg white	Water
30mins				
MOD	0.23±0.03	0.20±0.04	0.27±0.03	0.13±0.04
Mean difference		0.03	-0.04	0.09
t		2.86	-4.99	13.32
p value		**0.01**	**<0.001**	**<0.001**
CI		0.01-0.05	-0.06- -0.03	0.08-0.11
1 hour				
MOD	0.22±0.03	0.20±0.04	0.24±0.02	0.13±0.02
Mean difference		0.02	-0.01	0.1
t		1.90	-1.68	13.37
p value		0.06	0.1	**<0.001**
CI		-0.03–0.04	-0.03–0.03	0.08-0.11
3 hours				
MOD	0.22±0.03	0.15±0.04	0.22±0.08	0.12±0.02
Mean difference		0.07	0.00	0.10
t		7.22	0.00	14.85
p value		**<0.001**	0.93	**<0.001**
CI		0.05-0.09	-0.03–0.04	0.08-0.11

Intra-medium comparison of the mean optical densities of the cells revealed that, irrespective of the medium used, there was a decline in the viability of the cells within the first 30 minutes, and this was worse with cells stored in water. There was a statistically significant difference in the MOD of the cells at baseline compared with the MODs of the cells at all the other time points. Table 2 explains this.

Table 2. Intra-medium comparison of the mean optical densities of PDL cells in the different media over time

Media	F	p	Time	Mean difference	p	Confidence interval lower	upper
HBSS	75.75	<0.001	Baseline–30 mins	0.1	**<0.001**	0.07	0.13
			1 hour	0.1	**<0.001**	0.07	0.13
			3 hours	0.11	**<0.001**	-0.13	-0.14
			30 minutes–1 hour	0.00	1.00	-0.01	0.01
			3 hours	0.01	1.00	-0.01	0.01
			1 hour–3 hours	0.01	0.53	-0.01	0.01
Milk	106.52	<0.001	Baseline–30 mins	0.13	**<0.001**	0.10	0.15
			1 hour	0.12	**<0.001**	0.09	0.15
			3 hours	0.18	**<0.001**	0.15	0.20
			30 minutes–1 hour	-0.01	1.000	-0.03	0.02
			3 hours	0.05	**0.002**	0.02	0.09
			1 hour–3 hours	0.06	**0.001**	0.02	0.09
Egg white	31.69	<0.001	Baseline–30 mins	0.05	**<0.001**	0.03	0.08
			1 hour	0.09	**<0.001**	0.07	0.11
			3 hours	0.11	**<0.001**	0.07	0.15
			30 minutes–1 hour	0.04	**0.001**	0.01	0.06
			3 hours	0.06	**0.01**	0.01	0.10
			1 hour–3 hours	0.02	1.00	-0.02	0.07
Water	459.24	<0.001	Baseline–30 mins	0.19	**<0.001**	0.17	0.22
			1 hour	0.20	**<0.001**	0.17	0.23
			3 hours	0.21	**<0.001**	0.18	0.23
			30 minutes–1 hour	0.01	**0.02**	0.001	0.02
			3 hours	0.02	**<0.001**	0.01	0.02
			1 hour–3 hours	0.01	0.09	-0.001	0.01

Discussion

The viability of PDL cells in HBSS, evaporated milk, egg white and water was determined in this study using the MTT assay. This choice was based on its availability and superiority over the dye exclusion methods, as it measures mitochondrial integrity of the cells. The viability of the cells was presented as optical density as read by a spectrophotometer and this eliminates any inter- or intra-examiner variations[20]. This study recorded a decline in the viability of the cells in all the test media within the first 30 minutes of storage at room temperature. This corroborates the already known phenomenon, that immediate replantation of an avulsed tooth is the best treatment. The need for a transport medium arises when immediate replantation cannot be carried out[4].

Hanks Balanced Salt Solution has been used as a reference medium in studies on dental avulsion[13]. However, its inaccessibility has prompted the search for more readily available products, such as milk and egg white. At 30 minutes and 1 hour, it was observed that the cells in egg white had a higher viability than those in HBSS. Statistically significant difference was observed at 30 minutes and this was similar to the findings of Ulusoy et al.[29] (2016) . However, Khademi et al.[23] (2008) found no difference ($p>0.05$) between the viability of cells stored in egg white and those stored in HBSS, though the time interval was over a period of 12 hours. At 3 hours, the viability of the cells stored in egg white was found to be similar to that of the ones stored in HBSS and followed sequentially by those stored in milk and water. The choice of the use of skimmed milk over other types of milk in this study was based on the previous reports that it has the ability to preserve the viability of periodontal ligament cells better than whole milk owing to its low fat content[16]. However, according to the manufacturer, the brand of milk used in this study had 0.4% milk fat but also contained 7.6% vegetable fat, which may account for the poor performance of milk found in this study. Furthermore, it was observed that the viability of the cells stored in milk was lower than those stored in HBSS at all the study intervals, with a significant difference noted at 30 minutes and three hours. This agrees with the findings of Hwang et al.[18] (2011) and Ahangari et al.[19] (2013). However, some studies have reported higher viability of cells stored in milk relative to those stored in HBSS over a 24-hour study period[11,12,29]. The differences in methodology and variations in composition of milk used may account for the range of observations.

Water may be considered to be the most readily available storage medium for an avulsed tooth in any environment, but this study found that the cells stored in water had lower optical densities than those stored in HBSS ($p<0.05$) at all intervals. This is in congruence with previous reports[11,30] and may be due to the low osmolality of water, which results in cell lysis upon storage.

Intra-medium comparison of the viability of the PDL cells over time in all the studied media revealed that there was a statistically significant difference in the viability of the cells at baseline relative to other time points ($p<0.001$). This further emphasizes the need for immediate replantation of an avulsed tooth in its alveolar socket, which is its natural habitat, in order to keep optimal number of PDL cells viable.

This finding of similar viability levels all through the test period by the cells stored in HBSS in this study shows that, after the initial stabilization of the cells due to a change in environment, HBSS can maintain the viability of the remaining cells stored in it for up to 3 hours. For the cells stored in milk, their viability levels at 30 minutes and 1 hour were also similar, but there was decline in the cell viability between 1 and 3 hours ($p<0.05$). The latter observation may be due to a decline in the pH of milk over time[31]. However, the cells stored in egg white had a sequential decline in their viability, similar to the findings of Badakhsh et al.[20] (2014), and this accounted for a sequential decline in the nutrients in egg white over time.

In conclusion, this study found egg white to perform better than HBSS in supporting the viability of PDL cells up to 1 hour, with subsequent decline in its performance at 3 hours; while milk and water were less effective than HBSS in maintaining PDL cell viability. However, the latter observation may be due to the type of milk used in this

study. Hence, there is a need to determine the viability of PDL cells in other types of milk in this environment and also to check for viability of PDL cells in milk and egg white for longer period of time.

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability

Datasets related to this article cannot be shared because they are part of ongoing research.

Authors contributions

ATW, OOD and BOP participated in the initiation, conceptualization and reviewing of the manuscript while ATW, JAA, OMA, TEA participated in the conception of the methodology, laboratory work and reviewing of the manuscript. CAA participated in the statistical analysis and review of the manuscript. All authors have revised and approved the final version of the manuscript.

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