Viability and Reproducibility of Periodontal Ligament Cells on Avulsed Teeth Stored in Ham's F-10 Solution

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Objectives: *The purpose of the present study was to evaluate the efficacy of Ham's F-10 in maintaining the viability and reproducibility of PDL cells on avulsed teeth.* ***Study design:*** *Sixty mature, healthy extracted premolars were used. The experimental media used were Ham's F-10, Hank's balanced salt solution (HBSS), skim milk, and tap water (n = 15 specimens each). Cell viability was tested after 1, 3, 6, and 24 h storage in medium. Cell reproducibility was assessed by methyl-thiazol-tetrazolium (MTT) assay after1, 3, and 6 h storage in Ham's F-10, HBSS, and tap water.* ***Results:*** *The viability of PDL cells stored in Ham's F-10 and HBSS was significantly greater than that of samples stored in milk and tap water at all-time points (P<0.001). A significant difference in cell viability between samples stored in Ham's F-10 and HBSS (favoring the former) was observed only at 6h (P=0.04). MTT assay results were significantly better for samples stored in Ham's F-10 and HBSS than for those stored in tap water (P<0.001), with a significant difference between Ham's F-10 and HBSS observed only at 3h (P<0.001).*

Conclusions: *Ham's F-10 is capable of preserving PDL cells viable and reproducible better than milk and tap water and similar to HBSS.*

Key words: *avulsed tooth, Ham's F-10 cell culture medium, Hank's balanced salt solution, periodontal ligament cell, reproducibility, storage media, and viability.*

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INTRODUCTION

Avulsion injuries are among the worst types of dent alveolar injury, and extra oral time and storage conditions are the most crucial factors in determining the survivability of avulsed teeth after replantation [1-4]. Successful replantation also depends on the maintenance of periodontal ligament (PDL) cell vitality. When immediate replantation cannot be accomplished, avulsed teeth should be placed in a suitable storage medium. An ideal storage medium would be capable of preserving the viability, mitogenicity, and clonogenic capacity of the damaged PDL to facilitate repopulation of the denuded root surface, thereby preventing further root resorption. It should have physiological osmolality and pH, and be readily available or easily accessible at the site of an accident [5,6].

Storage media recommended for this purpose include Hank's balanced salt solution (HBSS), Eagle's medium, milk, Viaspan , Gatorade, Custodiol, propolis, egg white, saliva, normal saline, and tap water(Table1)(6).HBSS is a widely used standard solution recommended by the International Association of Dental Traumatology as a storage medium for avulsed teeth [7] .Viaspan, contact lens solution, and Gatorade on ice may serve as short-term storage media [8,9]. Cell viability in egg white and HBSS has similar results after 12 h storage time [10]. For longer-term storage of avulsed teeth, propolis and Viaspan are more effective than HBSS [11],

for an even longer storage period, soy milk and coconut water have resulted in greater viability than have HBSS and skim milk [12]. The efficacy of green tea in maintaining the viability of PDL cells is similar to that of HBSS and greater than that of milk [13].

Ham's nutrient mixtures (cell culture media) were originally developed to support growth of several clones of Chinese hamster ovary cells. Ham's F-10 medium has been shown to support the growth of human diploid cells, white blood cells for chromosomal analysis, and primary explants of rat, rabbit, and chicken tissues. Compared with other basal media, Ham's F-10 contains a wide variety of components, including zinc, hypoxanthine, thymidine, and enriched protein and growth factors[14,15].It has physiologic pH (7.2–7.4) and osmolality (280–300 mosmol/kg). It can be stored in powder form at room temperature for about 2 years, and its cost is similar to that of HBSS [14]. The purpose of the present study was to evaluate the efficacy of Ham's F-10 cell culture medium in maintaining the viability and reproducibility of PDL cells on avulsed teeth.

MATERIALS AND METHOD

This experimental study was conducted at Mashhad Dental Faculty, on approval obtained from Research Ethics Committee, Mashhad University of Medical Sciences. Sixty human permanent premolars with closed apices that had been extracted for orthodontic purposes were obtained. Extractions were performed as atraumatically as possible by a pediatric assistant. Following extraction, the teeth were held with forceps in the coronal region, and 3 mm coronal PDL was scraped with a curette to remove cells that may have been damaged. The teeth were rinsed three times with normal saline, and divided randomly into four experimental storage solution groups (n = 15 each): group 1 (study group), Ham's F-10 solution (N6908; Sigma-Aldrich, St. Louis, MO, USA); group 2 (positive control group), HBSS (H9394; Sigma-Aldrich); group 3, pasteurized skim milk (Kale Co., Amol, Iran); and group 4, tap water (negative control group). Each experimental tooth was soaked in storage medium in 15-ml Falcon tubes (BD Falcon, BD Biosciences, Bedford, USA). Teeth were transferred to the laboratory within 30 min (Fig 1).

Viability assay

The teeth were stored at room temperature for 1, 3, 6, and 24 h. After the assigned time, the teeth were treated with trypsin and collagenase enzymatic procedures. The supernatant was then removed with sterile micropipettes. After the addition of type 4collagenase, the trypsin procedure was performed. The cells were labeled with 0.4% trypan blue (gibco, Life Technologies, New York, USA) for the determination of viability. The number of viable PDL cells was counted under a light microscope with a hemocytometer at 40× magnification, then used in the viability formula (No. of viable cells counted/total cells counted (viable and dead) ×100=% viable cells).

Methyl-thiazol-tetrazolium assay

To determine cell proliferation in culture, the reproducibility of cells stored in Ham's F-10, HBSS, and tap water was determined at 1, 3, and 6 h bymethyl-thiazol-tetrazolium (MTT) assay [3-(A,5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazolium bromide; Sigma, St Louis, USA]. MTT assays were not performed on samples stored in milk because it is not a good substitute for serum in cell culture(16).Cells were seeded in 96-well micro plates at a density of 1×10^4 cells/well and incubated for 24 h to allow attachment. Thereafter, 100 µL MTT solution (0.5 mg/mL) was added and the cells were incubated in the dark for >4 h at 37°C in air containing 5% CO_2. Two positive and negative control groups' tests were also conducted to compare and assess cell viability of the product. The absorbance of the supernatant was then recorded at 540 nm using a microplate reader (EL x800; BioTek Instruments Inc., Winooski, USA). Cell numbers were determined using a standard curve that was established using a known cell number.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's honest significant difference tests were used to compare results among experimental groups. Repeated-measures ANOVA was used to determine cell viability. Bonferroni correction was performed to allow comparison of cell viability at different time points in each study group. Statistical significance was set at $P\leq0.05$.

RESULTS

Cell viability

Repeated-measures ANOVA showed that all storage protocols resulted in significant reduction of PDL cell viability over time (Table 2).At all-time points, cell viability was significantly greater in samples stored in Ham's F-10 and HBSS media than in those stored in milk and water. Tukey's test also showed a significantly greater number of viable PDL cells in samples stored in Ham's F-10 and HBSS media than in those stored in milk and water ($P<0.001$).Cell viability did not differ significantly between the Ham's F-10 and HBSS groups, except after 6h storage; at this time point, samples stored in Ham's F-10showed more viability than did those stored in HBSS ($P=0.04$).

Cell reproducibility

MTT assay results showed significant reductions in the percentage of reproducible PDL cells over time for all tested storage media (Table 3). Significantly better results were obtained for samples stored in Ham's F-10 and HBSS media than for those stored in water at all-time points($P<0.001$).A significant difference favoring Ham's F-10 over HBSS was observed only after3h storage ($P<0.001$).

Figure 1 Schematic diagram of the experimental design.

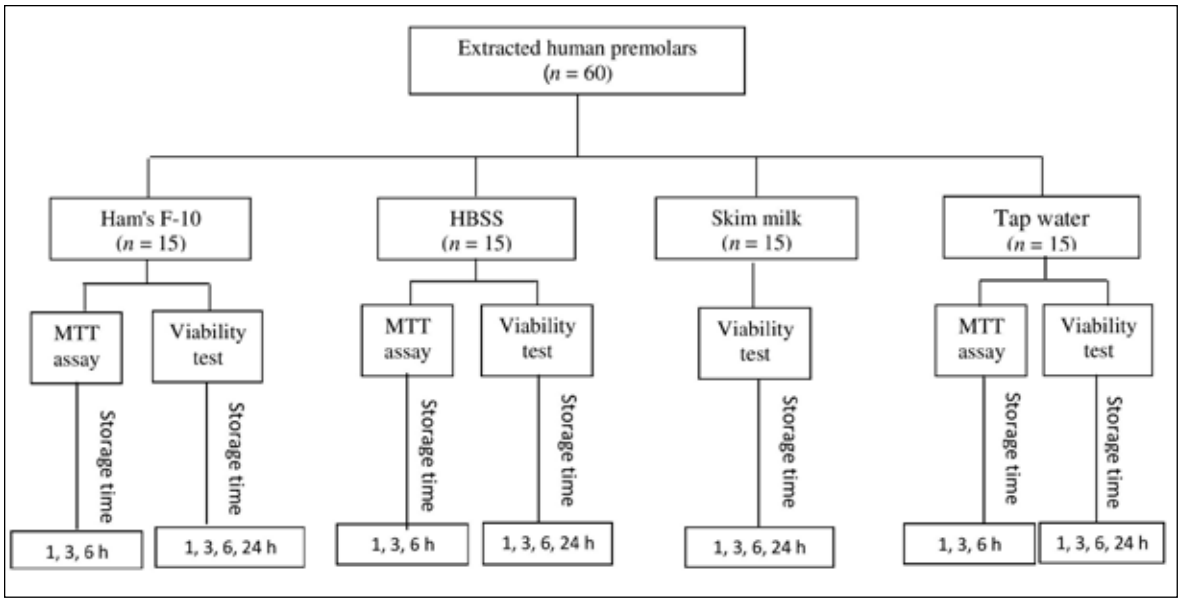

Table 1: Characteristics of storage media used in this study

Medium	pH	Osmolality(mosmol/kg)	Availability	Nutritional Content	Refrigeration	Cost Effectiveness	PDL Cell Viability	Mitogenic&-Clonogenic-Capacity
Hams F-10	7.0–7.6	271–299	Limited access at accident sites for first aid	Has essential metabolites	Not required	Similar to HBSS	Excellent	Excellent
HBSS	7.4	280	Limited access at accident sites for first aid	Has essential metabolites& glucose	Not required	Inexpensive	Excellent	Excellent
Milk	6.5–7.2	Similar to extracellular fluid(286)	Readily available	Has essential metabolites	Essential	Cost effective	Neither facilitates nor suppresses	Moderate
Water	7.4	30	Readily available	None	Not required	Cost effective	Cell lysis due to hypo tonicity	_

Table 2: The viability of periodontal ligament cells in storage media at different time points

Medium	Percentage of Viable Cells				*P*
	1 h	3 h	6 h	24 h	
Ham's F-10	91.27±4.75	81.6±8.08	68.87±9.07	49.2±13.68	<0.001*
HBSS	87.87±7.09	73.47±11.02	60.8±11.7	48.73±15.76	<0.001*
Milk	68.33±5.09	49.4±6.94	28.67±5.91	13.53±2.85	<0.001*
Water	49.80±8.47	32.93±9.83	13.47±2.85	6.20±2.54	<0.001*
P	<0.001*	<0.001*	<0.001*	<0.001*	

*Significant difference

Table 3: The reproducibility of periodontal ligament cells in various storage media at different time points

Medium	Percentage of Reproducible Cells			*P*
	1 h	3 h	6 h	
Ham's F-10	72.87±10.66	50.07±9.66	39.67±9.83	<0.001*
HBSS	74.67±11.33	61.33±9.36	37.6±12.00	<0.001*
Water	52.13±7.44	23.53±3.15	14.40±3.86	<0.001*
P	<0.001*	<0.001*	<0.001*	

* Significant difference

DISCUSSION

This study was performed to evaluate the use of Ham's F-10 as a storage medium for avulsed teeth by assessing the maintenance of PDL cell viability and reproducibility. Our results indicate that Ham's F-10 is as effective as HBSS (the gold standard) for the storage of avulsed teeth, likely due to its excellent biological proportionality. At all-time points, the viability of PDL cells stored in Ham's F-10 and HBSS media was significantly greater than those stored in milk and water. Ham's F-10 also supported PDL cell reproducibility well, similar to storage in HBSS and significantly superior to storage in water. Our results thus support the use of Ham's F-10 as a storage medium for avulsed teeth.

Differences in temperature (e.g., room temperature, body temperature, exposure to ice) can affect the viability of PDL cells [4,17]. As in other studies, all procedures were performed at clinical room temperature (25°C) in this study [8,18-21]. For short periods, body temperature is more ideal than room temperature [2]. Sigalas and Chamorro [5,8] showed that significantly more cells survived and proliferated when exposure occurred at temperatures below room temperature.

Various techniques have been used to quantify the number of viable PDL cells. To minimize the exposure of cells to active trypsin and preserve maximum cell viability, Gopikrishna [21], Sanghavi[19] and Pileggi [22]treated root surfaces with collagenase and grade II dispase. This procedure allowed rapid cell retrieval and maintained maximum cellular integrity. In the current study, as in that performed by Khademi [10], a stepwise trypsinization procedure followed by type IV collagenase and trypsin was used instead of dispase. This procedure was chosen because trypsin was readily accessible and is used specifically to separate fibroblastic cells, whereas dispase is used mainly to separate epithelial cells. As the use of trypsin for >20 min has harmful effects, we controlled the temperature (37°C) and exposure time (10–15 min). After this time, the enzyme was deactivated by the addition of growth culture at low temperature.

In this study, the trypan blue exclusion staining technique was used because it is quick, easily performed, and distinctively differentiates non-viable from viable cells [8,19] . However, the health of viable cells and their ability to proliferate cannot be determined with this technique. For this reason, we determined cell reproducibility by MTT assay.

Like HBSS solution and unlike Viaspan, milk, and Eagle's medium, Ham's F-10 does not require refrigeration. Its cost is similar to that of HBSS and much than those of Viaspan and Eagle's medium. However, Ham's F-10 medium is not ready available to the general public, unlike milk, egg, and saliva. If further studies prove the ability of Ham's F-10 to maintain the long-term (>24 h) viability and reproducibility of PDL cells, it should be produced in small containers for first-aid purposes and kept in public places where avulsion occurs, such as sports fields and schools.

This study has several limitations. MTT assays were not performed 24 h after extraction due to the inevitability of bacterial contamination of culture at room temperature. The performance of all steps at room temperature was not suitable for cell growth and proliferation. With the maintenance of PDL cells for more than 24 h, we probably would have observed greater differences between Ham's F-10 and HBSS due to the greater nutritional element and vitamin content of the former. Hence, further studies are needed to evaluate the ability of Ham's F-10 to maintain PDL cell viability and reproducibility over a longer time period.

CONCLUSION

This study indicated that not only Ham's F-10 cell culture medium are capable of preserving PDL cells viable and reproducible better than milk and tap water but it can also be easily accessed and used. Yet another advantage of this storage medium is that Ham's F-10 is not required to be kept in refrigerator which causes it to be accessible in the places where dental trauma may take place more frequently. So Ham's F-10 maybe a suitable, alternative storage medium for avulsed teeth, with effectiveness similar to that of HBSS.

REFERENCES

1.Andreasen JO AF, Andreasen L. Textbook and Color Atlas of Traumatic Injuries to the Teeth. 4thed. Oxford: Munksgaard Blackwell; 2007.

2.Macway-Gomez S, Lallier TE. Pedialyte promotes periodontal ligament cell survival and motility. J Endod 39(2):202-7,2013.

3.Lindskog S, Blomlof L. Influence of osmolality and composition of some storage media on human periodontal ligament cells. Acta Odontol Scand. 40(6):435-41, 1982.

4.Lekic PC, Kenny DJ, Barrett EJ. The influence of storage conditions on the clonogenic capacity of periodontal ligament cells: implications for tooth replantation. Int Endod J 31(2):137-40, 1998.

5.Chamorro MM, Regan JD, Opperman LA, Kramer PR. Effect of storage media on human periodontal ligament cell apoptosis. Dent Traumatol 24(1):11-6, 2008.

6.Udoye CI, Jafarzadeh H, Abbott PV. Transport media for avulsed teeth: a review. Aust Endod J 38(3):129-36, 2012.

7.Tavassoli-Hojjati S, Aliasghar E, Babaki FA, Emadi F, Parsa M, Tavajohi S, et al. Pomegranate juice (punica granatum): a new storage medium for avulsed teeth. J Dent (Tehran) 11(2):225-32, 2014.

8.Sigalas E, Regan JD, Kramer PR, Witherspoon DE, Opperman LA. Survival of human periodontal ligament cells in media proposed for transport of avulsed teeth. Dent Traumatol 20(1):21-8,2004.

9.Trope M, Hupp JG, Mesaros SV. The role of the socket in the periodontal healing of replanted dogs' teeth stored in ViaSpan for extended periods. Endod Dent Traumatol 13(4):171-5, 1997.

10.Khademi AA, Saei S, Mohajeri MR, Mirkheshti N, Ghassami F, Torabi nia N, et al. A new storage medium for an avulsed tooth. J Contemp Dent Pract 9(6):25-32, 2008.

11.Mori GG, Nunes DC, Castilho LR, de Moraes IG, Poi WR. Propolis as storage media for avulsed teeth: microscopic and morphometric analysis in rats. Dent Traumatol 26(1):80-5, 2010.

12.Moura CC, Soares PB, de Paula Reis MV, Fernandes Neto AJ, Zanetta Barbosa D, Soares CJ. Potential of coconut water and soy milk for use as storage media to preserve the viability of periodontal ligament cells: an in vitro study. Dent Traumatol 30(1):22-6,2014.

13.Hwang JY, Choi SC, Park JH, Kang SW. The use of green tea extract as a storage medium for the avulsed tooth. J Endod. 37(7):962-7, 2011.

14.Ham's Nutrient Mixtures 2008 [cited 2012 25 th july]. Available from: http://www.linscottsdirectory.com/products/united-states-biological/nutrient-mixture-ham-s-f-12-calcium-free-w-l-glutamine-powder-2.

15.RG H. An improved nutrient solution for diploid Chinese hamster and human cell lines. ExpCell Res. 29:515-26, 1963.

16.A E. Can milk replace serum in mammalian cell culture. Cytotechnology.8:1-4, 1992.

17.Schwartz O, Andreasen FM, Andreasen JO. Effects of temperature, storage time and media on periodontal and pulpal healing after replantation of incisors in monkeys. Dent Traumatol 18(4):190-5, 2002.

18.Gomes MCB, Westphalen VPD, Westphalen FH, Silva Neto U, Fariniuk LF, Carneiro E. Study of storage media for avulsed teeth. Brazilian Journal of Dental Traumatology. 1(2):69-76, 2009.

19.Sanghavi T, Shah N, Parekh V, Singbal K. Evaluation and comparison of efficacy of three different storage media, coconut water, propolis, and oral rehydration solution, in maintaining the viability of periodontal ligament cells. J Conserv Dent 16(1):71-4, 2013.

20.Ashkenazi M, Shaked I. In vitro clonogenic capacity of periodontal ligament fibroblasts cultured with Emdogain. Dent Traumatol 22(1):25-9, 2006.

21.Gopikrishna V, Thomas T, Kandaswamy D. A quantitative analysis of coconut water: a new storage media for avulsed teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 105(2):e61-5, 2008.

22.Pileggi R, Dumsha TC, Nor JE. Assessment of post-traumatic PDL cells viability by a novel collagenase assay. Dent Traumatol 18(4):186-9, 2002.