Atatürk Üniv Diş Hek Fak Derg (*J Dent Fac Atatürk Uni*)
Cilt:31, Sayı:3, Yıl: 2021, Sayfa: 362-6

Araştırma/ Research Article

EFFECT OF DIFFERENT AVULSED TOOTH STORAGE MEDIA ON *IN VITRO* CELL VIABILITY OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS

FARKLI AVULSE DİŞ SAKLAMA ORTAMLARININ *İN VİTRO* ORTAMDA İNSAN PERİODONTAL LİGAMENT FİBROBLASTLARININ HÜCRE CANLILIĞI ÜZERİNE ETKİSİNİN DEĞERLENDİRİLMESİ

Dr. Ögr. Üyesi Ahu DİKİLİTAŞ[*] **Dr. Ögr. Üyesi Fatih KARAASLAN**[*]
Prof. Dr. Mehmet TAŞPINAR[**] **Dr. Ögr. Üyesi Filiz TAŞPINAR**[***]

Makale Kodu/Article code: 4662
Makale Gönderilme tarihi: 04.11.2020
Kabul Tarihi: 07.04.2021
DOI : *10.17567/ataunidfd.915271*

Ahu Dikilitaş: ***ORCID ID:*** 0000-0003-4130-2526,
Fatih Karaaslan: ***ORCID ID:*** 0000-0002-9899-3316,
Mehmet Taşpınar: ***ORCID ID:*** 0000-0002-7598-2665
Filiz Taşpınar: ***ORCID ID:*** 0000-0003-3780-4021

ABSTRACT

Aim: An optimum storage medium is required to preserve the viability of the periodontal ligament cells of the avulsed tooth. Although it has been suggested by some researchers that it would be suitable to store the avulsed tooth in water or saliva, milk has been considered the most appropriate storage medium. Although milk has been shown to be more effective for long durations, short-term results are contradictory. Thus, the purpose of this study was to assess *in vitro* capacity of four storage media at room temperature with mitochondrial function analysis to maintain the viability of periodontal ligament fibroblasts over different time periods.
Material and Methods: Periodontal ligament samples were isolated from second premolars for extracted orthodontic reasons. Pasteurized long-life milk, saliva, tap water, and distilled water were used to examine periodontal ligament cell viability. The exposure periods were for 60, 120, and 240 min and cell viability were evaluated using the XTT assay.
Results: The count of living cells in milk was found to be significantly higher than in other storage media after 60, 120, and 240 minutes. The mean number of viable cells after 240 min in all storage media except distilled water was significantly lower than the average number of viable cells after 60 min ($p<0.05$, t-test).
Conclusion: Although milk is considered to be a suitable storage medium for preserving avulsed teeth, the count of vital cells is adversely affected in long-term storage.
Key words: cell viability, fibroblast, avulsed tooth, milk**.**

ÖZ
Amaç: Avulse olan dişin periodontal ligament hücrelerinin canlılığının korunması için uygun bir saklama ortamı gerekmektedir. Bazı araştırıcılar tarafından avulse dişin suda ya da tükürükte saklanmasının uygun olacağı önerilse de süt en uygun saklama ortamı olarak kabul edilmiştir. Saklama ortamları arasında sütün diğer saklama ortamlarına göre uzun sürede daha etkili olduğu gösterilmiş olsa da kısa vadeli sonuçlar çelişkilidir. Bu çalışmanın amacı, farklı zaman dilimlerinde periodontal ligament fibroblastlarının canlılığını korumak için dört depolama ortamının *in vitro* kapasitesini oda sıcaklığında mitokondriyal fonksiyon analizi ile değerlendirmektir.
Gereç ve yöntem: Periodontal ligament fibroblastları; ortodontik nedenlerle çekilen ikinci premolar dişlerden elde edilmiştir. Periodontal ligament hücrelerinin canlılığını incelemek için pastörize uzun ömürlü süt, tükürük, musluk suyu ve damıtılmış su kullanılmıştır. Dişin depolama ortamında bekleme süreleri 60, 120 ve 240 dakikadır ve hücre canlılığı XTT testi kullanılarak değerlendirilmiştir.
Bulgular: Sütteki canlı hücre sayısı 60, 120 ve 240 dakika sonra diğer depolama ortamlarına göre anlamlı derecede yüksek bulunmuştur. Canlı hücre sayısı damıtılmış su dışındaki tüm depolama ortamlarında 240 dakika sonra, 60 dakika sonraki tüm depolama ortamlarından anlamlı derecede düşük bulunmuştur ($p<0.05$, t-testi).
Sonuç: Süt avulse dişin saklanması için uygun bir ortam olmasına rağmen, dişin uzun süreli saklanmasında canlı hücrelerin sayısı negatif etkilenmektedir.
Anahtar kelimeler: hücre canlılığı, fibroblast, avulse diş, süt.

[*]Department of Periodontology, Faculty of Dentistry, Usak University, Usak.
[**] Department of Medical Biology, Faculty of Medicine, Aksaray University, Aksaray.
[***] Department of Physiology, Faculty of Medicine, Aksaray University, Aksaray.

Kaynakça Bilgisi: Dikilitaş A, Karaaslan F, Taşpınar M, Taşpınar F. Farklı avulse diş saklama ortamlarının *in vitro* ortamda insan periodontal ligament fibroblastlarının hücre canlılığı üzerine etkisinin değerlendirilmesi. Atatürk Üniv Diş Hek Fak Derg 2021; 31: 362-6.
Citation Information: Dikilitas A, Karaaslan F, Taspinar M, Taspinar F. *Effect of different avulsed tooth storage media on in vitro cell viability of human periodontal ligament fibroblasts. J Dent Fac Atatürk Uni 2021; 31: 362-6.*

INTRODUCTION

Although immediate replantation is the most favorable condition for survival of an avulsed tooth, it is not possible in most cases.[1] The presence of living PDL fibroblast cells is very important for the long-term success of replantation, which plays an essential role in minimizing future complications such as ankylosis and root resorption.[2,3] Dry storage and longer extra-alveolar time are less favorable in maintaining PDL cell viability.[4]

In previous studies, the optimum storage medium for preserving the viability of PDL fibroblast cells was investigated for an avulsed tooth.[3-6] It is necessary to have a storage medium that can maintain cell viability, has the necessary nutrients, neutral pH, physiological osmolarity, and is also easily accessible.[5] Although some researchers recommend keeping the avulsed tooth in water or in saliva, milk has been widely accepted as a storage medium due to its low bacterial content and its ability to provide certain nutrients and growth factors to the cells.[6]

Studies have shown that an avulsed tooth can be repositioned within 60-180 minutes without complications after placement in proper storage conditions.[7] Although milk has been shown to be more effective than other storage media for long periods of time, short-term results are contradictory.[4,8] For this reason, the goal of this study was to assess the *in vitro* efficiency of four different storage media for maintaining the viability of PDL fibroblasts at different time periods by analysis of mitochondrial functioning at room temperature.

MATERIALS AND METHODS

Patients were fully informed about the aim of the study, and signed consent forms were obtained in line with the Ethics Committee Report of Yuzuncu Yıl University (05.12.2013-05).

Sample collection

PDL samples were isolated from second premolars for extracted orthodontic reasons. The teeth were atraumatically and aseptically extracted under local anesthesia following oral prophylaxis procedure.

Preparation of PDL cell cultures

Extracted teeth were washed with 1 mL of phosphate buffered solution (PBS) (pH 7.4) followed by rinsing with 0.5 mL of antibiotic solution (a mixture of 100 mg/mL of penicillin, 100 mg/mL of amphotericin B, and 100 mg/mL of streptomycin). To obtain Fibroblast tissue samples, the middle third of the root was scraped using Bard Parker blade #15 (GPC Medical Limited, New Delhi, India).

The tissue samples were located in a sterile petri dish containing Dulbecco's Modified Eagle Medium (DMEM) solution with 10% fetal bovine serum. Disaggregation of PDL tissue was done by using 0.2% collagenase and 0.125% trypsin for 30 min, and the cells were accumulated by centrifugation for 5 min. Supernatant was discarded, and cell pellet which settled at the bottom was collected. The cells were resuspended and incubated with 5% CO2 for attachment. The cells were trypsinized and passed into T25 culture flasks. The cells of passages 3–5 were trypsinized and pooled for experimentation. The count of vital cells in each batch was calculated by Neubauer hemocytometer and batches showing more viability were used for the experiment.

Exposure of PDL cells to various storage media

The trypsinized cells were plated in 96-well culture plates (TPP, Trasadingen, Switzerland) and incubated at 37 C with 5% CO2 for 24 h. The medium in the wells was replaced with pasteurized long-life milk, saliva, tap water, and distilled water. Six replicates were used to examine each test solution. The exposure periods were 60, 120, and 240 min. The cell viability was calculated using the XTT assay. The absorbance was assessed at 480 nm by a UV-Vis microplate reader (Biochrom, Cambridge, UK).

Filtered saliva

Each individual's own saliva, collected at least 1.5 hours after eating, drinking and brushing teeth, was used. Unstimulated saliva samples were centrifuged and sterilized with a 0.22 µm filtration system (Stericup.1, Millipore, Bedford, MA, USA).

Statistical analysis

Version 17.0 of SPSS was used for data analysis. At the end of 60, 120, and 240 min, the difference between the count of vital cells in the media was examined by ANOVA test. The change between the count of vital cells in the media at the end of 60 and 240 min was examined by dependent t-test.

RESULTS

The number of viable cells in milk was significantly higher than that of other storage medium at the end of the 60 min., 120 min. and 240 min. time

period ($p<0.05$, Anova test) (Table 1-2-3). There was no significant difference between tap water and distilled water at the end of the 60 min time period ($p>0.05$, Anova test) (Table 1).

The average number of viable cells after 240 min in all storage media except distilled water was significantly lower than the average number of viable cells after 60 min ($p<0.05$, t-test) (Table 4).

Table 1. The number of viable cells at the end of the 60 min

Storage media (60 min)	n	Mean±sd	F	p
Distilized water	6	0.17±0.06	**1222.089**	**0.001***
Tap water	6	0.14±0.02		
Milk	6	2.96±0.02		
Saliva	6	0.69±0.09		

*: $p<0.05$, ANOVA test

Table 2. The number of viable cells at the end of the 120 min

Storage media (120 min)	n	Mean±sd	F	p
Distilized water	6	0.14±0.01	**249.38**	**0.001***
Tap water	6	0.12±0.01		
Milk	6	2.53±0.31		
Saliva	6	.45±0.08		

*: $p<0.05$, ANOVA test

Table 3. The number of viable cells at the end of the 240 min

Storage media (240 min)	n	Mean±sd	F	p
Distilized water	6	0.12±0.01	**484.12**	**0.001***
Tap water	6	0.10±0.01		
Milk	6	2.05±0.12		
Saliva	6	0.34±0.05		

*: $p<0.05$, ANOVA test

Table 4. The difference between viable cells at the end of the 60 min and 240 min

Storage media (60 min-240 min)	n	Mean±sd	t	p	Decrease (%)
Distilized water (60 min)	6	0.17±0.06	2.037	0.097	-
Distilized water (240 min)	6	0.12±0.01			
Tap water (60 min)	6	0.14±0.02	**3.699**	**0.014***	-25.3
Tap water (240 min)	6	0.10±0.01			
Milk (60 min)	6	2.96±0.02	**17.322**	**0.001***	-30.8
Milk (240 min)	6	2.05±0.12			
Saliva (60 min)	6	0.69±0.09	**9.195**	**0.001***	-51.4
Saliva (240 min)	6	0.34±0.05			

*: $p<0.05$, t test

DISCUSSION

At the end of all time periods, the number of viable cells in milk was statistically higher than that of other media. Saliva was determined to be the second best medium for maintaining PDL cell viability. Tap water and distilled water were determined to be the least effective for maintaining cell viability.

In accordance with previous studies, our results showed that milk is superior in maintaining PDL cell viability when compared with other transport media after 60–240 min. In the study by Oikarinen and Seppā, no difference was found between milk and saliva after a 60 minute incubation.[9-11] The reason can be explained by the proliferation capacity of PDL cells, which does not decrease more significantly in saliva than in milk during shorter incubation periods. In this study, milk can be suggested as a transport medium for short-term preservation because of its low bacterial content, physiological osmolarity, neutral pH, and nutrients and factors that will ensure PDL cell viability.[12]

According to this *in vitro* study, saliva was less suitable than milk but more effective than tap water. Non-physiological pH, high bacterial contamination, and hypotonicity are unfavorable characteristics of saliva that makes it less suitable than milk.[13,14] Although saliva is not effective enough in maintaining cell viability, it still is more preferable than keeping the tooth in a dry environment or in tap water.

The charactersitcs of tap water are inadequate for being used as storage medium. In line with the outcome of this study, many studies identify that cells cannot maintain their morphological continuity in tap water, which has bacterial contamination and non-physiological pH.[15,16]

Although distilled water is boiled, sterilized and purified by the thermal phase change process, its behavior in maintaining cell viability is not statistically different from that of tap water. This can be explained by the idea that PDL cell viability is related to osmolarity rather than to chemical structure or bacterial load of the storage media, as stated by Bomlof et al.[17] Although distilled water is more pure and sterile than tap water, its osmolarity is similar.[15]

When the count of vital cells at last 60 min and 240 min incubation periods are compared, a decrease in the count of vital PDL cells was found to be the greatest in saliva (50.1%), followed by milk (30.8%), and tap water (25.3%). The decreasing effectiveness of saliva in maintaining PDL viability can be associated with the failure of saliva to meet the energy and nutrient requirements of the cells, as well as with the presence of microorganisms in saliva.[18,19] The reason of the decrease in cell number over time in milk may be due to the gradual decrease in pH of milk, which creates an unsuitable environment for cells to

survive.[20,21] Although a significant decrease was observed in the number of living cells in tap water over time, no significant change was observed in cell viability in distilled water. This may be because tap water is exposed to greater bacterial contamination than is distilled water, and the mineral and ion exchange required for cell viability is minimal in distilled water.[22]

The viability of PDL cells in this study was evaluated at room temperature. Although there are some studies indicating that more successful results are obtained when avulsed teeth are stored in low-temperature media, it is stated that avulsed teeth are mostly stored at room temperature.[16,23] Therefore, room temperature was preferred as ambient temperature, in accordance with the typical clinical environment.[24]

Many methods are used to identify viable PDL cells in the dental literature. The choice of one test over another depends on a variety of factors, including the limitations of each test, ease of use, speed, and available resources. In this study, the XTT method was used to determine viable cell numbers. It is also reported that the sensitivity of the XTT reduction assay is better than MTT assay, and it reduces assay time in many viability assay protocols.

This study has some limitations. First, although tooth extractions were performed under atraumatic and aseptic conditions, any trauma that may occur during tooth extraction may affect PDL cell viability. Second, the XTT method was used to determine PDL viability. Apart from this method, other cell culture models can be used to determine cell viability.[25,26]

CONCLUSION

Although milk and saliva are the better storage media in all time periods, the highest decrease in the count of viably cells with time was seen in these media. Milk and saliva are seen as suitable storage media for preserving avulsed teeth, whereas the count of viable cells is adversely affected in long-term storage.

ACKNOWLEDGEMENTS
The authors report no conflicts of interest related to this study and the study did not receive any financial support.

REFERENCES

1. Lou Q, Zhu Y, Wang X. Fourteen Years After Delayed Replantation of an Avulsed Permanent Tooth: Clinical Features and Outcomes. J Craniofac Surg 2019;30:692-4.
2. Souza BDM, Dutra KL, Kuntze MM, Bortoluzzi EA, Flores-Mir C, Reyes-Carmona J, et al. Incidence of root resorption after the replantation of avulsed teeth: a meta-analysis. J Endod 2018;44:1216-27.
3. Kaya S, Keskin Ö, Bodrumlu E. Storage solutions of avulsed teeth. J Dent Fac Atatürk Uni 2013;Suppl 7:96-101.
4. Khinda VI, Kaur G, Brar GS, Kallar S, Khurana H. Clinical and practical implications of storage media used for tooth avulsion. Int J Clin Pediatr Dent 2017;10:158.
5. Dharmani CKK, Singh N, Dharmani UK. Storage Media for Avulsed Teeth: An Overview. J Dent Med Sci 2017;16:142.
6. Adnan S, Lone MM, Khan FR, Hussain SM, Nagi SE. Which is the most recommended medium for the storage and transport of avulsed teeth? A systematic review. Dent Traumatol 2018;34:59-70.
7. Martin MP, Pileggi R. A quantitative analysis of Propolis: a promising new storage media following avulsion. Dent Traumatol 2004;20:85-9.
8. Kokkali V, Bendgude V, Sharangpani G. Comparative evaluation of post-traumatic periodontal ligament cell viability using three storage media. Eur Arch Paediatr Dent 2017;18:209-14.
9. D'Costa VF, Bangera MK, Kini S, Kutty SM, Ragher M. An In vitro comparison of coconut water, milk, and saline in maintaining periodontal ligament cell viability. J Pharm Bioallied Sci 2017;9:107-11.
10. Côvre LM, Caliente EA, Sonoda CK, Brandini DA, Poi WR. The effect of soy and whole milk as a means to store avulsed teeth: A histometric study. Dent Traumatol 2020;00:1-9.
11. Oikarinen KS, Seppā ST. Effect of preservation media on proliferation and collagen biosynthesis of periodontal ligament fibroblasts. Dent Traumatol 1987;3:95-9.
12. Souza BDMd, Alves AMH, Santos LGPd, Simões CMdO, Felippe WT, Felippe MCS. Fibroblast Viability after Storage at 20° C in Milk, Hank's Balanced Salt Solution and Coconut Water. Braz Dent J 2016;27:404-7.

13. Goswami M, Chaitra T, Chaudhary S, Manuja N, Sinha A. Strategies for periodontal ligament cell viability: An overview. J Conserv Dent 2011;14:215-20.
14. Malhotra N. Current developments in interim transport (storage) media in dentistry: an update. Br Dent J 2011;211:29-33.
15. De Souza BDM, Bortoluzzi EA, Da Silveira Teixeira C, Felippe WT, Simões CMO, Felippe MCS. Effect of HBSS storage time on human periodontal ligament fibroblast viability. Dent Traumatol 2010;26:481-3.
16. Souza BDM, Lückemeyer DD, Felippe WT, Simões CMO, Felippe MCS. Effect of temperature and storage media on human periodontal ligament fibroblast viability. Dent Traumatol 2010;26:271-5.
17. Blomlof L. Milk and saliva as possible storage media for traumatically exarticulated teeth perior to replantation. Swed Dent J 1981:1-26.
18. Cvikl B, Lussi A, Moritz A, Sawada K, Gruber R. Differential inflammatory response of dental pulp explants and fibroblasts to saliva. Int Endod J 2016;49:655-62.
19. Müller H-D, Cvikl B, Lussi A, Gruber R. Chemokine expression of oral fibroblasts and epithelial cells in response to artificial saliva. Clin Oral Investig 2016;20:1035-42.
20. de Souza BD, Lückemeyer DD, Felippe WT, Alves AM, Simões CM, Felippe MC. Effect of milk renewal on human periodontal ligament fibroblast viability in vitro. Dent Traumatol 2012;28:214-6.
21. Lee HS, Lim YS. Comparative study on survival rate of human gingival fibroblasts stored in different storage media. J Korean Soc Dent Hyg 2012;12:733-9.
22. Moazami F, Mirhadi H, Geramizadeh B, Sahebi S. Comparison of soymilk, powdered milk, Hank's balanced salt solution and tap water on periodontal ligament cell survival. Dent Traumatol 2012;28:132-5.
23. de Souza BDM, Bortoluzzi EA, Reyes-Carmona J, dos Santos LGP, Simões CMdO, Felippe WT, et al. Effect of temperature and seven storage media on human periodontal ligament fibroblast viability. Dent Traumatol 2017;33:100-5.
24. Chen F, Qi S, Lu L, Xu Y. Effect of storage temperature on the viability of human periodontal ligament fibroblasts. Dent traumatol 2015;31:24-8.
25. Nowak E, Kammerer S, Küpper J-H. ATP-based cell viability assay is superior to trypan blue exclusion and XTT assay in measuring cytotoxicity of anticancer drugs Taxol and Imatinib, and proteasome inhibitor MG-132 on human hepatoma cell line HepG2. Clin Hemorheol Microcirc 2018;69:327-36.
26. Wang S, Yu H, Wickliffe JK. Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale TiO2. Toxicol In Vitro 2011;25:2147-51.

Sorumlu Yazarın Yazışma Adresi
Ahu Dikilitaş,
Department of Periodontology,
Faculty of Dentistry, Usak University,
Usak Turkey.
Phone: +90 0506 356 95 00
E-mail: ahu.dikilitas@usak.edu.tr