

Comparative Evaluation of Corneal Storage Medias Used as Tooth Avulsion Medias in Maintaining the Viability of Periodontal Ligament Cells Using the Cell Counting Kit-8 Assay

Nidhi James¹, Sandya Kini¹, Swathi Pai¹, Neetha Shenoy¹, Shama Prasada Kabekkodu²

¹Department of Conservative Dentistry and Endodontics, MCO DS, MAHE, Manipal, Karnataka, India; ²Department of Cell and Molecular Biology, School of Life Sciences, MAHE, Manipal, Karnataka, India

Correspondence: Sandya Kini, Department of Conservative Dentistry and Endodontics, MCO DS, MAHE, Manipal, Karnataka, 576104, India, Tel +91 9886823982, Email sandya.kini@manipal.edu

Purpose: The prime factor in determining the success of reimplantation of an avulsed tooth is the maintenance of the viability of periodontal ligament fibroblast cells (PDFC). This study aims to evaluate and compare Mc Carey Kaufman media (MK), Cornisol, Dulbecco's Modified Eagles Medium (DMEM), Hanks Balanced Salt Solution (HBSS) and distilled water in preserving the viability of the PDFC using the Cell Counting Kit-8 assay (CCK-8).

Methods: Cryopreserved PDFC were suspended in DMEM and incubated in CO₂ incubator at 37°C with 95% humidity and 5% CO₂ for attachment. Once cells attained 80% confluence, they were trypsinised and passed into T-25 culture flasks to expand the culture population. Cells from passage 5 were pooled for experimentation. Trypan blue exclusion test was performed before each experiment to measure cell viability and batches showing more than 95% viability were used in the experiment. The viable PDFC with 1×10⁵ were seeded in 96 well plates and incubated in CO₂ incubator at 37°C, 95% humidity and 5% CO₂ for 24 hours to allow cell attachment. A 100µL of the experimental media were added in the wells and the cells were exposed for 1, 24 and 48 hours respectively. The viability was determined using the CCK-8. Experiment was performed in triplicates and data was subjected to statistical analysis.

Results: Statistical analysis was performed using repeated measure ANOVA, ANOVA, and post-hoc Bonferroni test with the significance level p<0.05. The values are as follows: MK (1.3146 ±0.0588, 1.9012±0.0511, 2.0723±0.1211) > Cornisol (1.2399 ±0.0548, 1.9596±0.0652, 1.9592±0.1361) > DMEM (1.1914±0.0691, 1.8479±0.0116, 2.0718±0.0795) > HBSS (0.3665±0.0814, 0.0184 ±0.0010, 0.0248±0.0042) > distilled water (0.0122±0.0033, 0.0225±0.0085, 0.0104±0.0008) at 1 hour, 24 hours and 48 hours respectively. MK > Cornisol > DMEM > HBSS > distilled water.

Conclusion: It can be concluded that the corneal preservation solutions showed promising results in preserving periodontal ligament cell viability for extended time periods.

Keywords: McCarey Kaufman media, Cornisol, DMEM

Introduction

The oral cavity is a small region compared to the rest of the body but it still holds up to 5% of all the bodily injuries.¹ Among them, the incidence of tooth avulsion is up to 0.5–16% of total dental injuries.² World Health Organization described tooth Avulsion or Exarticulation as the complete displacement of a tooth from its socket owing to trauma.² Following an avulsion, there are many changes seen in the vasculature of the periodontium causing hypoxic changes, which causes detrimental effects on the cellular metabolism and eventually impedes the recovery of periodontal ligament fibroblast cells (PDFC). Singh et al, in their study explained the importance of the viability of periodontal cells following tooth avulsion, and mentioned that it is critical to support the survival of PDFC adhered to the avulsed tooth.³ Immediate

tooth reimplantation is the best method to manage tooth avulsion with a high success rate. However this is not always possible especially in cases with other life-threatening injuries. Hence preserving the avulsed tooth in a suitable storage media helps to maintain the viability of PDFC.⁴

Among the different storage media such as Eagle's culture medium, Viaspan, milk, Propolis, green tea, egg albumin, coconut water, saline, distilled water, etc. HBSS (Hanks Balanced Salt Solution) is considered superior regarding the preservation of cell viability. It is non-toxic, has a balanced pH, and osmolality.⁵⁻⁷ However for developing countries, the cost, and easy accessibility are a matter of concern. Additionally HBSS promotes osteoblastic differentiation of PDFC. Hence the search for an avulsion media continues. Cornisol and Mc Carey Kaufman media (MK) are corneal preservation solutions used in ophthalmology for maintaining the survival of corneal cells. Studies have shown some similarities between PDFC and corneal cells at the cellular level and share similar neural crest cell origin and proteoglycan secretion.⁸ Cornisol is a corneal storage media that is used to preserve the human corneas indicated for keratoplasty up to a time period of 14 days at a temperature of (2–8°C). It is made of Chondroitin Sulphate which helps in maintaining the integrity of the cell membrane by acting as a membrane stabilizer, recombinant human insulin which acts as an enhancer of metabolism and dextran which functions as an osmotic agent along with stabilized L glutamine, ATP precursors, vitamins, trace elements, gentamicin, streptomycin, and pH indicators. Singh et al evaluated and compared the efficacy of Cornisol, HBSS, and normal saline in maintaining the viability of PDFC and concluded that the percentage of cell viability of Cornisol and HBSS using MTT assay is comparable at a short time duration (30 min) but at 1, 24, 48 and 96 h, Cornisol is superior to HBSS.³ McCarey-Kaufman medium is also a corneal storage media, first reported in 1974, was developed by McCarey and Kaufman hence the acronym "MK."⁹ MK is composed of Dextran 40 (< 1% w/w), MK Base Powder (< 1% w/w), HEPES Buffer (< 1%w/w), Sodium Bicarbonate (< 1%w/w), Phenol Red (< 1%w/w), Gentamycin (< 1%w/w), Purified Water (Balance).¹⁰ The medium had an osmolality of 290 mOsm and pH 7.4.⁹ The results of the clinical studies using MK to preserve corneas were good when stored for up to 96 hours.^{11,12}

MTT assay, Trypan blue staining, Alamar blue, Prestoblue assay, and Cell Counting Kit-8 assay (CCK-8) are commonly used assays for measuring cell viability and proliferation rate.¹³ CCK-8 is a one-bottle solution that provides easy, fast, dependable, and sensitive values for checking the viability of cells.¹³ Cell Counting Kit-8 uses a tetrazolium salt, WST-8, which produces the water soluble WST-8 formazan. This is reduced by active metabolic cells via dehydrogenase enzymes. The reduction product obtained is colored and its concentration is determined by colorimetric measurements.^{13,14}

There are no known studies to the best of our knowledge that evaluates and compares MK and Cornisol with the routinely used tooth avulsion media. Hence our study aims to evaluate the effectiveness of MK, Cornisol, HBSS, Dulbecco's Modified Eagle Medium (DMEM) and Distilled water as storage media in maintaining the periodontal ligament fibroblast cell viability at 1 hour, 24 hours and 48 hours using CCK-8 assay. Studies have shown that replantation of an avulsed tooth has been done at various time periods that ranges from 1 hour up to 48 hours. Hence three time periods were selected for this study.^{3,15}

Materials and Methods

Ethical clearance was obtained by the, Manipal Academy of Higher Education (MAHE) Ethical clearance committee with ethical clearance no 750/2018.

Cell Culture

All the experiments were performed using periodontal ligament fibroblast cells (PDFC). The isolation, characterization and maintenance of PDFC's were performed as described by Singh et al.³ The fibroblast cells were cultured in DMEM containing 10% Fetal Bovine Serum (Himedia, India) at 37°C, 95% humidity, 5% CO₂ in a Heracell CO₂ Incubator (ThermoFisher Scientific, USA). The cells were maintained in a T-25 cell culture dish (Greiner Bio-One, India). Upon reaching 70–80% confluency, the cells were trypsinized using 0.05% trypsin containing 0.53mm EDTA solution and used for downstream experiments. All the experiments were performed using passage 5 cells.

Trypan Blue Staining

This test is based on the principle that a living cell has an intact cell membrane that prevents the uptake of any dye whereas a dead cell takes up the dye as the cell membrane is not intact, hence with this test the dead cells will take up the blue stain from trypan whereas the viable cells show a clear cytoplasm. The cell viability upon trypsinization was evaluated by trypan blue dye exclusion method as published by Strober.¹⁶ In brief, the cells harvested by trypsinization were exposed to 0.4% trypan blue dye (1:1 ratio). The stained cells were immediately loaded into hemocytometer and cells were counted using a CKX41 microscope (Olympus, Japan). The unstained transparent cells and cells stained with blue color were considered as live and dead cells, respectively. The total number of viable cells per mL of aliquot were calculated by multiplying the viable cells with 2 (i.e. the dilution factor). The percentage of viable cells are calculated using the formula.

$$\text{Viable cells(\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100$$

Exposure of PDFC to Storage Medias

The effect of different media on fibroblast cell viability was tested by CCK-8 assay. In brief, fibroblast cells were harvested from 70–80% confluent T-25 flask by standard trypsinization procedure and cell viability was tested by trypan blue staining method. Subsequently, 8000 fibroblast cells were seeded in a 96 well plate in DMEM containing 10% FBS. The cells were incubated overnight at 37°C, 95% humidity, 5% CO₂ in a Heracell CO₂ Incubator. Subsequently, the media was discarded, washed with Phosphate buffer solution and the cells were exposed to 100 microliter each of MK (L.V Prasad Eye Institute, Hyderabad, India), Cornisol (Aurolab, Tamil Nadu, India), DMEM (Himedia Laboratories Pvt. Ltd, Bangalore, India), HBSS (Phoenix-Lazerus, Inc, Shartlesville, PA, USA) and distilled water (KMC Pharmacy, Manipal, India), keeping HBSS as a positive control and distilled water as the negative control. After 1hr, 10 microliter of CCK-8 (Dojindo Molecular Technologies, Japan) was added to each well, and incubated for 4 hr. The 96 well plate was scanned using an Infinite M200 microplate reader (Tecan, Switzerland) at 450 nm. Similarly, after 24 hours and 48 hours. CCK-8 was added and the cells were incubated for 4 hours and later checked for cell viability (Figure 1). All the

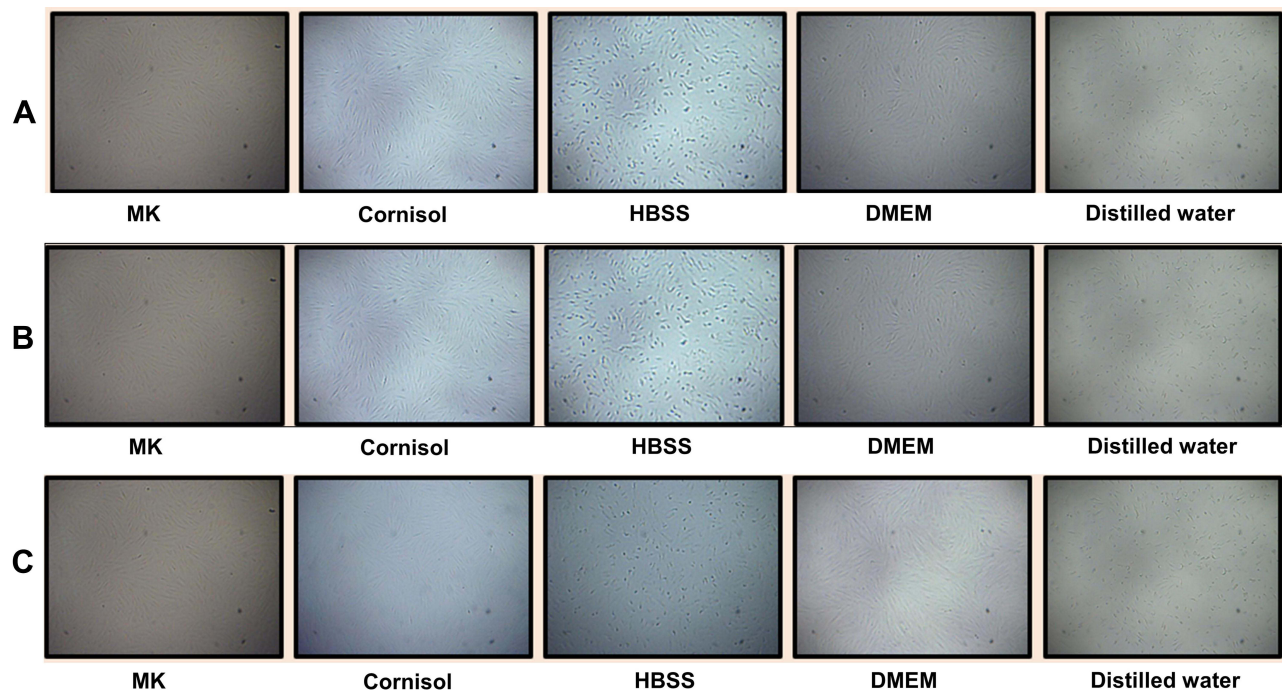


Figure 1 The cell viability of Mc Carey Kaufman media (MK), Cornisol, Hanks Balanced Salt Solution (HBSS), Dulbecco's Modified Eagles Medium (DMEM) and distilled water as seen under inverted microscope at 10 X magnification using WST-8 reagent by CCK-8 assay. (A–C) denotes the cell viability at 1 hour, 24 hours and 48 hours respectively.

experiments were performed in triplicates and repeated thrice. We have used the following formula to calculate the percentage of cell viability.

$$\text{Survival rate(\%)} = \frac{A_{\text{sample}} - A_{\text{b}}}{A_{\text{c}} - A_{\text{b}}} \times 100$$

wherein A_{sample} , A_{b} , and A_{c} , are absorbance/Optical density of fibroblast exposed to test reagents, absorbance/Optical density of blank, negative control, respectively.

Statistical Analysis

SPSS version 20 (IBM, USA) software was used for all the statistical analysis. The level of significance was set at less than or equal to 0.05. Comparison of the viability of PLC's at different time intervals within the group was done using Repeated Measure ANOVA, ANOVA, and POST-HOC BONFERRONI TEST, and Comparison of the viability of PLCs between different groups at each interval was done using POST-HOC BONFERRONI TEST.

Results

Viability was higher at 48 hours for DMEM group (2.0718 ± 0.0), at 1 hour in HBSS (0.3665 ± 0.0814), at 24 hours in Cornisol group (1.9596 ± 0.0652), at 48 hours in MK (2.0723 ± 0.1211), at 24 hours in Distilled Water group (0.0225 ± 0.0085). Repeated measures ANOVA was applied to compare the viability of cells at different time intervals within the group. Statistically significant difference was seen with respect to DMEM group ($p=0.001$), HBSS group ($p=0.018$), Cornisol ($p=0.011$) and MK ($p=0.003$) whereas there was no significant difference seen with respect to Distilled Water ($p=0.187$) (Table 1).

DMEM group showed significant difference between 1 hour and 48 hours, 24 and 48hours, HBSS and Cornisol group showed significant difference between 1 hour and 24 hours, 1 hour and 48 hours; MK showed significant difference between 1 hour and 24 hours ($p \leq 0.05$) whereas there was no difference between any time intervals with respect to Distilled Water group (Supplementary Table 1).

At 1 hour, MK showed highest viability of periodontal ligament cells (1.3146 ± 0.0588); at 24 hours, Cornisol showed highest viability of periodontal ligament cells (1.9596 ± 0.0652); at 48 hours, MK showed highest viability of periodontal ligament cells (2.0723 ± 0.1211). ANOVA was applied to compare the groups at different time intervals. ANOVA test showed statistically significant difference among the groups at 1 hour ($p < 0.001$), 24 hours ($p < 0.001$) and at 48 hours ($p < 0.001$) (Supplementary Table 2).

Statistically significant difference was seen between DMEM and HBSS, DMEM and Distilled Water, HBSS and Cornisol, HBSS and MK, Cornisol and Distilled Water, MK and Distilled Water at all the time intervals whereas there was no difference between DMEM and MK, Cornisol and MK media at all the time intervals. There was a statistical significant difference seen between DMEM and Cornisol only at 24 hours (Supplementary Table 3).

Discussion

Hammer in 1995 was the first person to demonstrate the importance of the viable PDFC for a successful treatment outcome of tooth avulsion.¹⁷ In cases where immediate reimplantation of the tooth is not possible, it is advised to store the tooth in a media that provides an environment which helps in maintaining the viability of the PDFC until the tooth is reimplanted back in its socket.¹⁷ Based on the severity of other associated injuries, the duration of the tooth reimplanted into the socket can vary. The best option is to store the avulsed tooth in a suitable media to maintain the viability of PDFC until the patient can seek emergency care and the tooth is reimplanted into the socket.

Lekic et al, suggested that a lower time period has an increased clonogenic capacity of PDFC and is also associated with the PDFC progenitor cells ability to attach and recolonize the root surface after reimplantation.¹⁸ On the other hand, Andreasen et al in 1995 suggested that even if the teeth have been kept dry for an extended duration, it should be replanted.¹⁹ A similar case was done by Tolentino et al, where an avulsed tooth was successfully replanted two days (48 hours) post the trauma.²⁰ Hence the effectiveness and efficacy of storage media were evaluated from 1 hour to 48 hours respectively.

Table I Comparison of the Viability of the Periodontal Ligament Cells at Different Time Intervals Within the Group Using Repeated Measures ANOVA

Groups	Time Intervals	Min	Max	Mean	SD	Repeated Measures ANOVA	p value
DMEM	1 hour	1.1320	1.2673	1.1914	0.0691	141.81	0.001*
	24 hours	1.8345	1.8554	1.8479	0.0116		
	48 hours	1.9887	2.1471	2.0718	0.0795		
HBSS	1 hour	0.2849	0.4476	0.3665	0.0814	52.95	0.018*
	24 hours	0.0178	0.0196	0.0184	0.0010		
	48 hours	0.0200	0.0276	0.0248	0.0042		
Cornisol	1 hour	1.1767	1.2746	1.2399	0.0548	51.83	0.011*
	24 hours	1.8896	2.0186	1.9596	0.0652		
	48 hours	1.8445	2.1096	1.9592	0.1361		
MK	1 hour	1.2540	1.3714	1.3146	0.0588	52.80	0.003*
	24 hours	1.8422	1.9319	1.9012	0.0511		
	48 hours	1.9326	2.1476	2.0723	0.1211		
Distilled water	1 hour	0.0094	0.0159	0.0122	0.0033	3.81	0.187
	24 hours	0.0175	0.0323	0.0225	0.0085		
	48 hours	0.0096	0.0112	0.0104	0.0008		

Note: *Denotes statistically significant with $p < 0.05$.

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; MK, Mc Carey Kaufman media; HBSS, Hanks Balanced Salt Solution.

Corneal preservation solutions MK and Cornisol were used in our study because of the similarity between PDFC and corneal cells in developmental pathways. Seo et al in their study showed that periodontal ligament stem cells (PDLSCs) and Dental Pulp Stem Cells (DPSC) originate from the cranial neural crest and also share similar developmental pathways as Corneal Stroma Keratocytes (CSK).²¹ Syed-Picard et al, illustrated that Dental Pulp Stem Cells can differentiate into CSK-like cells expressing keratocan (KERA), a unique marker for CSKs, and Keratan sulphate proteoglycans (KSPG).²² Various studies suggest that PDLSCs have clonal proliferation capability and express specific markers of Mesenchymal Stem Cells (MSCs), embryonic stem cells, and Neural crest-derived stem cells.²¹ Yam GH et al., suggested that corneal and PDLSCs share similar extra-cellular matrix components such as collagen type II, collagen type V, collagen type VI, Matrix metalloproteinases (MMP1 and MMP3).²³ Considering all these factors we expected better results while using these solutions as tooth storage media.

Trypan blue exclusion method was used in the present study to the maintain viable cell uniformity between all the triplicates and avoid any kind of bias in the study.³ MTT, MTS, CCK-8 and the resazurin-based reagents such as Prestoblue, alamar blue are assays used to check the viability of cells. However, the metabolic activity of cells used in Prestoblue assays, can reduce resazurin during the storage. With high cell numbers and prolonged storage, over-reduction of resazurin may occur and produce an uncolored, nonfluorescent product causing artefacts.²⁴ CCK-8 assay

was used in our study because it is one of the sensitive and reliable colorimetric assay for the determination of the cell viability.¹³ It is very stable and it has little cytotoxicity and shows sensitivity better than other tetrazolium salts like MTT, XTT, or MTS. It also shows good repeatability, detection speed, and stability as compared to other assays. Another advantage for CCK-8 is that it is ready-to-use, one-bottle solution that does not require any preparation and also does not require any redissolving of the solution.^{14,25} In the CCK-8 assay, the WST-8 reagent is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells.

HBSS, Save a Tooth was used as a positive control because it is recommended by the International Association of Dental traumatology as a medium for avulsed tooth.³ Distilled water was taken as a negative control in our study, which is in accordance with other studies where water as a negative control showed the lowest efficacy in maintaining the viability of the cells.^{7,26–28}

Both the corneal solutions showed similar results in our study which could be because both have similar properties such as pH and osmolarity and the presence of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) which, unlike conventional buffer, is superior in maintaining physiological pH despite the changes in CO₂ concentration caused by cellular respiration. As mentioned by Pereira et al, in their study, dextran, a high molecular weight polymer of glucose is used as an osmotic agent in both Cornisol and MK media.²⁹ The osmotic retention of water by dextran present in tissues may also be the reason for maintaining the wellbeing of the viable PDFCs.

MK media has a pH of 7.4, which is a favorable pH for the maintenance of PDFC viability. It also has an osmolarity of 290 mOsm, which falls into the range of the accepted requirement of a storage media to be used for an avulsed tooth. As reported by Lindstrom RL., the passive bicarbonate flux remained virtually undisturbed in MK which suggests its superior maintenance of the endothelial barrier for the survival of the cells. The gentamycin antibiotic present in MK would have helped in the keeping the microbial count low.³⁰

Cornisol has also shown similar results with MK. This could be because of the presence of chondroitin sulfate, which is the unique and key component of Cornisol. It maintains cell membrane integrity due to its antioxidant properties.³⁰ At lower temperatures, chondroitin sulfate brings about favorable effects on the cell membrane structure. Hence, Cornisol enhances Endothelial and Epithelial cell preservation, also in a study by Parekh et al, the presence of an antibiotic (mixture of streptomycin and gentamycin) (100 units/mL) in Cornisol was suggested to prevent microbial contamination of corneal cells.³¹

DMEM has shown similar results to MK media and Cornisol. This could be attributed to its high quantity of vitamins, amino acids, and glucose.³² This is also in accordance with the study conducted by Singh et al which shows the similar performance of Cornisol with DMEM.³ Cornisol has shown superior results at 24 hours compared to DMEM, which suggests that Cornisol is a better medium compared to DMEM if extraoral dry time is 24 hours. However, DMEM cannot be used as a storage media clinically to preserve an avulsed tooth because as mentioned by Casaroto et al, because the medium has to be freshly prepared, also with the increased release of CO₂ the pH also increases. Hence the pH of this medium should be monitored from time to time.³³ This would not be feasible in a clinical scenario.

HBSS comprises of inter alia glucose and sodium, potassium ions, with the Ph of 7.2–7.3 and 270–290 mOsm/kg. HBSS has shown lower values compared to Cornisol, MK media at all time periods, suggesting that MK and Cornisol are better storage media.³ HBSS has shown promising result up to 1 hour and a significant drop at 24–48 hours. Save-A-Tooth that was commercially available was used in our study may have shown lower effectiveness as compared to freshly prepared HBSS, additionally the pH of Save-A-Tooth solution is 6.4, which may have resulted in lower cell viability.³⁴ This is in accordance with the study by Souza et al and Singh et al suggesting that Cornisol and MK are better tooth storage media when the reimplantation of tooth would be delayed beyond 1 hour.^{3,34}

HBSS has shown similar results to Distilled water at 24 and 48 hours suggesting that this media would not be useful if the tooth reimplantation procedure would be delayed. This is also confirmed by the study conducted by Olson et al, wherein the optical density of the cells kept in Save-A-Tooth at a time period of 8 and 12 hours showed comparable values to the cells kept in a dry environment.³⁵ Distilled water has shown the least viability as compared to all the other

media which is in accordance with studies done by Casaroto et al and Andreasen JO. This is because, water is hypotonic in the intracellular environment, exhibits increased microbial contamination, and has a pH and osmolarity above the physiological limit which results in lysis of cells.^{27,33}

Conclusion

Within the preliminary data obtained in our study, it can be concluded that the corneal preservation solutions showed promising results in preserving periodontal ligament cell viability for extended time periods. With increasing time period corneal preservation solutions would be a better option compared to HBSS.

Disclosure

The authors report no conflicts of interest in this work.

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