ORIGINAL RESEARCH

An *In Vitro* Evaluation of Ice Apple as a Novel Storage Medium to Preserve the Viability of Human Periodontal Ligament Fibroblasts

Samhita Bijlani[1], Raghavendra S Shanbhog[2]

Abstract

Aim: For a better prognosis of an avulsed tooth, it is important to store it in an appropriate storage medium prior to replantation. The purpose of this study was to evaluate the ability of ice apples to preserve the viability of periodontal ligament (PDL) fibroblasts.

Materials and methods: Periodontal ligament (PDL) fibroblasts were procured from the roots of healthy premolars and cultured in Dulbecco's Modified Eagle's Medium (DMEM). They were preserved with—ice apple water (IAW), 30 and 10% ice apple fruit pulp extract (IAFPE), DMEM, negative control without any agent, and positive control DMEM supplemented with fetal bovine serum (FBS). Culture plates were incubated with investigational media at 37°C for 1, 3, and 24 hours. Each experiment was repeated thrice. Assessment of cell viability was done using 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay. After every test period, storage media was removed; 60 µL of MTT solution was added to each well and incubated for 3 hours at 37°C. The supernatant was aspirated and the formazan blue crystals formed were solubilized using dimethyl sulphoxide (DMSO) (100 µL). Optical density was measured at a wavelength of 490 nm. The effects of the test storage media at each time period were evaluated by a one-way analysis of variance (ANOVA) test, and this was followed by *post hoc* Tukey's multiple comparison tests ($p < 0.05$).

Results: A total of 10% IAFPE demonstrated the maximum capacity to maintain PDL cell viability in all three test periods ($p = 0.001$). Among the different forms of ice apple used in this study, IAFPE showed better results as compared to IAW ($p = 0.001$).

Conclusion: Ice apple fruit pulp extract (IAFPE) at 10% concentration showed the maximum capacity to maintain PDL cell viability across all three test periods. Therefore, it can be considered a suitable alternative natural storage medium for avulsed teeth. However, further, more detailed studies are required in this field.

Keywords: Cell survival, Ice apple, Periodontal ligament fibroblasts, Storage media, Tooth avulsion.

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Introduction

Tooth avulsion is the total displacement of the tooth out of its alveolar socket due to traumatic dental injury.[1] It is characterized by injury to the periodontal tissue and disruption of the neurovascular supply of the affected tooth.[2] The primary goal in the management of such injuries is to preserve the viability of the PDL cells in order to prevent the complications associated with delayed replantation. According to Andreasen et al., delayed replantation is when replantation is carried out beyond 5 minutes of avulsion.[3] Therefore, the most recommended approach in the management of an avulsed tooth is its instant replantation. However, in the majority of these cases, the replantation of an avulsed tooth is delayed, which leads to a poor long-term prognosis. The most prevalent complication after replantation is ankylosis, followed by external root resorption.[4] When immediate replantation is not feasible, the avulsed tooth must be placed in an appropriate storage medium in order to maintain any remaining vitality before it can be replanted. The ideal storage medium should be easily accessible, should be able to preserve cell viability, and must have essential nutrients, physiological osmolality, antioxidant properties, a neutral pH, adherence, and clonogenic capacity. A number of different synthetic and natural products have been studied for maintaining PDL cell viability. So far, the most studied and approved storage media have been Hank balanced salt solution (Gibco, Waltham, Massachusetts, United States of America), which has favorable biological properties, and milk, which is easily accessible.

[1,2]Department of Pediatric & Preventive Dentistry, JSS Dental College & Hospital, JSS Academy of Higher Education & Research (Deemed to be University), Mysuru, Karnataka, India

Corresponding Author: Raghavendra S Shanbhog, Department of Pediatric & Preventive Dentistry, JSS Dental College & Hospital, JSS Academy of Higher Education & Research (Deemed to be University), Mysuru, Karnataka, India, Phone: +91 9945112080, e-mail: drraghu.kiddoc@gmail.com

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A number of studies have demonstrated the ability of coconut water to preserve PDL cell viability.[5–8] Considering the nutritional properties of coconut water, it has been recommended as a good medium for the storage of avulsed teeth. Ice apple (*Borassus flabellifer*) is a locally available fruit in coastal regions of India. It is the juicy and fleshy fruit of the sugar palm tree, found during

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summertime. Botanically coconut water and ice apples are the same. Both are endosperms of seeds, where coconut water is the endosperm in liquid form, whereas ice apples are endosperms in semi-solid form. The fresh pulp of this fruit is known to be rich in vitamins A and C. It is rich in several phytochemicals that possess strong antioxidant and anti-inflammatory properties, dietary fiber, and minerals. The composition of coconut water is quite similar to that of the intracellular fluid, which makes it a suitable medium to maintain the vitality of PDL cells. Both are packed with rehydrating properties, minerals, vitamins, and fibers and have a cooling effect. Since ice apple and coconut water have similar characteristics, ice apple could be evaluated as a storage medium for avulsed teeth.

The aim of the present study was to evaluate the effectiveness of IAW and IAFPE in preserving the PDL cell viability in contrast with positive and negative control at 1, 3, and 24 hours.

Materials and Methods

Study Design and Location

This was an *in vitro* experimental type of study. The study protocol was reviewed and approved by the Institutional Research Ethics Committee prior to the commencement of the study (JSSDCH IEC Research Protocol no: 14/2018).

Preparation of Test Storage Solutions

Collection of IAW

The ice apple fruit is borne in clusters and covered with a black husk. The top portion of the fruit was removed to uncover the translucent pale-white jelly seed sockets. These jelly seeds are present in groupings of two to four seeds inside the fruit. This jelly part of the fruit comprises a watery fluid inside and is covered with a thin, yellowish-brown skin. The IAW was collected by inserting an 18-gauge needle into the jelly part of the fruit, through the yellowish-brown skin, and aspirating the fluid. It was collected in a sterile syringe and then filtered using syringe filters (0.45 μm).

Preparation of IAFPE

This extract was obtained by preparing fresh juice of the gelatinous portion. Under a sterile hood, the sweet jelly seeds were carefully removed from the hard covering. Thin, yellowish-brown skin covering the jelly part of the fruit was then removed. Thus, obtained jelly part of the fruit was triturated to make a homogenous mix. This homogenous mix was filtered using sterile syringe filters (0.45 μm) to obtain IAFPE and was stored in a sterile tube. At the time of the experiment, 30 and 10% IAFPE were prepared by diluting in DMEM solution.

Cell Culture

The PDL cell culture experimental model was used in this study. Cells were procured from healthy human teeth extracted for clinical necessity. The coronal portion of the tooth was held using forceps, and with the use of a #15 scalpel blade, the PDL was scraped. This tissue was then transferred to sterile Petri dishes and washed with DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL), and fungizone (5 mg/mL) in a humidified atmosphere containing 5% CO_2 at 37°C. Then, it was split into smaller pieces before transferring into tissue culture flasks. These pieces of tissue were maintained in the supplemented DMEM and allowed to stick to the surface of the flask in an atmosphere with 5% CO_2 at 37°C. Renewal of the culture medium was done two times in 1 week till the time the cells reached confluence. Then, using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid for 3–5 minutes, the cells were detached from the culture flasks using and sub-cultivated. A master bank and a working bank of cells were prepared using the first and second passages of cells, respectively. Further, these cells were suspended in a cryopreservation medium, which contained 20% FBS and 10% DMSO. The culture flasks were stored frozen in liquid nitrogen.[2]

Before starting the experiment, cells of the third and fourth passages were thawed at 37°C and seeded into the culture plates.

The cell-seeding into the 96-well plate was done at a density of 104 cells/well, and incubated for 2 hours in a CO_2 incubator at 37°C.

On the day of the experiment, this culture medium was removed from each of the wells, and the cells were subjected to 200 μL of the test storage solutions:

- IAW
- The 30% IAFPE
- A 10% IAFPE
- Negative control (NC)–without any agent
- DMEM
- Supplemented DMEM—DMEM + FBS

The 96 wells of microtiter plates were incubated for 1, 3, and 24 hours in a CO_2 incubator at 37°C.

Determination of Cell Viability

To evaluate the viability of PDL cells, an MTT assay was performed. *In vitro* viability and growth proliferation of cells were assessed by spectrophotometric determination of conversion of MTT into "formazan blue" by living cells.

A 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay is a colorimetric assay that measures the decline of yellow MTT by mitochondrial succinate dehydrogenase. This MTT penetrates the mitochondria in the cells and is reduced to an insoluble, dark purple-colored formazan crystal. After incubation for 3 hours, the cells are solubilized with an organic solvent. Thus, the released, solubilized formazan product is measured spectrophotometrically. The viability of cells is measured by the level of activity of the cells since the reduction of MTT occurs only in metabolically active cells.

After every test period, the test storage media was removed from the wells. About 60 μL of MTT solution was added to each of the wells and incubated at 37°C for 3 hours. The supernatant was aspirated, and the precipitated crystals of "formazan blue" were solubilized by adding DMSO (100 μL).

The optical density was measured at a wavelength of 490 nm by using an iMark™ Microplate Reader. The result recorded is the mean of three readings. Each experiment was repeated three times.

Formula

Surviving cells (%) = [Mean optical density (OD) of test agent × 100]/ Mean OD at control.

Statistical Analysis

Statistical analysis was carried out using Kolmogorov–Smirnov one-sample test for normality and the Levene test for homoscedasticity (same variances). Results displayed the normal distribution of the data, and homogenous variances were observed ($p < 0.05$). The effects of storage solutions at each time point were evaluated by a ANOVA test followed by *post hoc* Tukey's test. A type I error rate of 0.05 was used as the statistical significance threshold.

Results

The obtained data were analyzed using Statistical Package for the Social Sciences-PC v 24. Arithmetic mean and standard deviation (SD) were used for descriptive statistics.

The pH of storage media was assessed using a Mettler Toledo Benchtop pH meter. pH values are depicted in Table 1.

Table 2 represents the percentage of viable PDL cells for each media and the storage periods tested. The mean percentage of viable cells at 1 hour was maximum in the 10% IAFPE group (153.67%). This was followed by 30% IAFPE having a mean percentage of 144.33% of viable cells. The lowest mean percentage of viable cells value was shown by the NC group, which was 63.33%. On comparing all test media, a statistically significant difference was observed (p-value = 0.001). After 3-hour exposure also, 10% IAFPE displayed the maximum mean percentage of viable cells, that is, 117.33%. NC group showed a mean percentage of viable cells value at only 55.33%. When all these values were compared, there was a statistically significant variation noted (p-value = 0.001). After 24 hours, 10% IAFPE again showed the highest mean percentage of viable cells at 97.33%. This was followed by 30% IAFPE with 96.33% viable cells. However, IAW displayed a statistically significantly lower mean percentage of viable cells, at 89.33%. The NC group had only 30.67% of cells viable at the end of 24 hours. The variation observed when all these were compared was statistically significant (p-value = 0.001).

Table 3 represents the comparison of the percentage of viable cells of the different test storage media compared with each other at 1 hour. The results revealed that there was a statistically significant variation in all the media when they were compared with each other (p-value = 0.001).

Table 1: pH of test storage media

Storage media	*pH*
IAW	5.33
IAFPE	5.43

Table 4 represents the multiple comparison analysis of the percentage of viable cells at 3 hours. When the different test storage media were compared with each other, the mean difference in the values of the percentage of viable cells indicated that DMEM + FBS and 30% IAFPE did not show any statistically significant difference when compared against each other. Likewise, there was no statistically significant difference between 30% IAFPE and IAW. However, 30% of IAFPE and IAW showed statistically significant mean differences.

Table 5 represents the comparison of the percentage of viable cells of the different storage media at 24 hours. The values of the mean difference in the percentage of viable cells in these tables showed that 30% IAFPE, 10% IAFPE, and DMEM + FBS did not have statistically significant mean difference values. However, among these two subsets, the difference in the mean scores of IAW compared with 30% IAFPE, 10% IAFPE, and DMEM + FBS displayed statistically significant variation.

Discussion

An avulsion is a complex injury characterized by loss of attachment of the PDL cells. As a result, the nutrient supply for the survival of the PDL cells is lost. The survival of the remaining PDL on the surface of the avulsed tooth depends on the supply of vital metabolites. It is highly critical to keep these cells attached to the PDL viable for a better prognosis of the treatment. The survival of these cells will initiate the production of new cells, which will restore the supporting tissues. The main viewpoint of this survival involves the inhibition of protein synthesis in the cell. The recovery of the PDL following injury is encouraged by the action of fibroblasts and the healing of connective tissue.[5]

Hammer in 1955, was the first to demonstrate how important PDL cell viability is before replanting an avulsed tooth. He proved that the amount of viable periodontal membrane determines the extent of survival of the replanted tooth.

The healing of the PDL represents the primary consequence when the treatment options for tooth avulsions are assessed. The

Table 2: Mean percentage of viable cells at different time intervals

Test Period	*Storage media*	*Mean*	*SD*	*Sum of squares*	*Mean square*	*F*	*p*
1 hour	IAW	129.67	3.512	24459.33	2717.70	440.70	0.001***
	30% IAFPE	144.33	2.517				
	10% IAFPE	153.67	2.517				
	NC	63.33	3.055				
	DMEM	81.67	1.155				
	DMEM+FBS	100.00	0.000				
3 hours	IAW	106.67	2.082	12440.00	1382.22	351.41	0.001***
	30% IAFPE	101.33	1.155				
	10% IAFPE	117.33	2.309				
	NC	55.33	0.577				
	DMEM	78.00	1.000				
	DMEM+FBS	100.00	0.000				
24 hours	IAW	89.33	0.577	11260.96	1251.21	695.12	0.001***
	30% IAFPE	96.33	1.155				
	10% IAFPE	97.33	0.577				
	NC	30.67	1.528				
	DMEM	76.67	2.082				
	DMEM+FBS	100.00	0.000				

*** Highly significant

Table 3: Multiple comparison analysis of the percentage of viable cells at 1 hour

Storage media	*Mean deviation (MD)*	*p*
IAW vs 30% IAFPE	14.666	0.001***
IAW vs 10% IAFPE	24.000	0.001***
IAW vs NC	66.333	0.001***
IAW vs DMEM + FBS	29.666	0.001***
30% IAFPE vs 10% IAFPE	9.333	0.010**
30% IAFPE vs NC	81.000	0.001***
30% IAFPE vs DMEM + FBS	44.333	0.001***
10% IAFPE vs NC	90.333	0.001***
10% IAFPE vs DMEM + FBS	53.666	0.001***
NC vs DMEM + FBS	36.666	0.001***

** Moderately significant; *** highly significant

Table 4: Multiple comparison analysis of the percentage of viable cells at 3 hours

Storage media	*MD*	*p*
IAW vs 30% IAFPE	5.333	0.010
IAW vs 10% IAFPE	10.666	0.001***
IAW vs NC	51.333	0.001***
IAW vs DMEM + FBS	6.666	0.002
30% IAFPE vs 10% IAFPE	16.000	0.001***
30% IAFPE vs NC	46.000	0.001***
30% IAFPE vs DMEM + FBS	1.333	0.810
10% IAFPE vs NC	62.000	0.001***
10% IAFPE vs DMEM + FBS	17.333	0.001***
NC vs DMEM+FBS	44.666	0.001***

*** Highly significant

Table 5: Multiple comparison analysis of percentage of viable cells at 24 hours

Storage media	*MD*	*p*
IAW vs 30% IAFPE	7.000	0.001***
IAW vs 10% IAFPE	8.000	0.001***
IAW vs NC	58.666	0.001***
IAW vs DMEM + FBS	10.666	0.001***
30% IAFPE vs 10% IAFPE	1.000	0.689
30% IAFPE vs NC	65.666	0.001***
30% IAFPE vs DMEM + FBS	3.666	0.047
10% IAFPE vs NC	66.666	0.001***
10% IAFPE vs DMEM + FBS	2.666	0.036**
NC vs DMEM + FBS	69.333	0.001***

** Moderately significant; *** highly significant

most recommended treatment strategy is immediate replantation of the tooth. However, this is not always probable. Therefore, it is best that the avulsed tooth is temporarily stored in a capable storage medium that has the potential to preserve PDL cell viability before seeking the necessary assistance for replantation. This medium should have the required properties to supply nutrients to the PDL cells for survival. This survival depends not only on the nutrients but also on the storage time and pH of the medium. An ideal medium should be readily available, have minimal or no microbial contamination, and have a suitable pH for maintaining PDL cell viability.[6] Various natural and synthetic storage media have been recommended as storage media for avulsed teeth.

The present study aimed at evaluating and comparing the efficacy of a new agent, ice apple, as a storage medium for avulsed teeth.

Ice apple is a local fruit available in the coastal regions of India during summertime. It is the juicy, fleshy fruit of the sugar palm tree known to be packed with rehydrating properties and a cooling effect.

The results of this *in vitro* experiment at 1 hour showed that 10% IAFPE had 153.67% viable cells (mean OD = 0.570), thus displaying the highest capacity to maintain cell viability. Whereas 30% IAFPE showed 144.33% (mean OD = 0.536) of viable cells, indicating that a lower concentration of IAFPE was statistically significantly more effective than a higher concentration of the same extract ($p = 0.001$). Since this is the first study that has been conducted to evaluate the efficacy of ice apples, there are no previous comparative studies for the same.

The MTT test reveals the cell viability and cell proliferation capacity of the media since the formazan that is formed is proportional to the active mitochondrial enzymes.[7] The values of this test revealed an increase in the number of viable cells in these media, indicating their proliferative capacity on PDL cells.

After 3-hour exposure to the storage media, 10% IAFPE still showed the maximum percentage of viable cells, with 117.33% viable cells (mean OD = 0.440). Therefore, it indicated that 10% IAFPE continued maintaining the viability of cells and also had some proliferative capacity for up to 3 hours. At this time interval, IAW displayed 106.67% viable cells (mean OD = 0.422), which was statistically significantly lesser as compared to 10% IAFPE ($p = 0.001$) and 30% IAFPE ($p = 0.021$) at 3 hours. Considering both these products are obtained from the same source, it is reasonable to say that the extract of ice apple fruit performed statistically significantly better ($p = 0.001$) than the natural water of ice apple in maintaining PDL cell viability.

By the end of 24 hours of exposure of PDL cells to the test storage media, it was clearly apparent that 10% IAFPE with 97.33% viable cells (mean OD = 0.458) was the best in maintaining the viability of PDL cells among the other test media. IAW and 30% IAFPE, showed a relatively lower percentage of viable cells. Over the course of 24 hours, IAFPE underwent oxidation and appeared darker in color. This color change could have affected the optical density value of these test media. A blank OD value of test agents was taken before the start of the experiment. To compensate for the variation in OD, the blank values of 30 and 10% IAFPE (0.352 and 0.327) were subtracted from the observed OD values (0.806 and 0.785) to obtain the final result (0.454 and 0.458).

The lowest concentration of IAFPE used in this present study was most effective in maintaining PDL cell viability. The pulp of this fruit is rich in vitamins, iron, zinc, potassium, calcium, and phosphorus. Every 100 gm of edible pulp contains 0.8 gm protein, 10.9 gm carbs, 1 gm fiber, 27 mg calcium, 0.1 gm fat, 1 mg iron, 0.04 mg thiamine, 87.6 gm water, 0.02 mg riboflavin, 0.3 mg niacin, 5 mg vitamin C, and 30 mg phosphorous.[8] These constituents are similar to those present in coconut water. Coconut water has already been proven as an efficient storage medium for avulsed teeth. Hence, it is rational to conclude that the results obtained by testing ice apples are in agreement with those obtained in the studies conducted on coconut water.[9–13]

Within the limitations of the present study, it can be concluded that IAFPE at 10% of its concentration showed the maximum

capacity to maintain PDL cell viability when stored for 1, 3, and 24 hours. Therefore, as ice apple showed promising results in maintaining PDL cell viability, it can be considered a suitable alternative natural storage medium for avulsed teeth. However, further more detailed *in vitro*/*in vivo* studies are required in this field.

Orcid

Samhita Bijlani https://orcid.org/0000-0002-4447-0923

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