Viability of Human Periodontal Ligament Fibroblasts After Time in Tooth Storage Media

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VIABILITY OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS AFTER TIME IN TOOTH STORAGE MEDIA

By

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ABSTRACT

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Objective:

Periodontal ligament cell viability at the time of tooth replantation is of utmost importance for proper repair of the damaged tissues (1). Multiple different options are currently available for storage of an avulsed tooth. The goal of this study was to investigate new storage media and evaluate their ability to maintain cell viability over time.

Materials and Methods:

Cultured human periodontal ligament fibroblasts (hPDLF) were seeded on 96-well plates. These cells were exposed to different storage media including: Hank's Balanced Salt Solution (HBSS), EMT Toothsaver (EMT), Save-A-Tooth (SAT), trehalose (T300), cell culture media, water, and different formulations of HBSS combined with trehalose (HBSST150, HBSST50) and cell culture media with trehalose (mediaT150, mediaT50). Experimental groups were tested at both room temperature (22°C) and refrigeration (4ºC). Cell viability results were read at 1, 2, 6, 12, 24, and 48 hours.

Results:

At room temperature, mediaT150, mediaT50, and media performed the best out of all the experimental groups but only mediaT150 was significantly better than the next best storage media, HBSS. T300 performed better than SAT, EMT and water but was only significantly better than water. SAT and EMT were not significantly different from water. At refrigerated temperature the best performing storage media included mediaT150, HBSST150, mediaT50, media, and HBSST50. The next highest performing group included T300 and HBSS which were not significantly different from HBSST50 and media. SAT was found not significantly different from EMT but was better than water. EMT was the worst performing experimental group and had no significant difference from water.

Conclusion:

Cell culture media in combination with 150mM trehalose had a significantly better performance than HBSS as a storage media, especially over the first 12 hours. The current commercially available storage media (SAT and EMT) were not significantly different from water at room temperature. Room temperature storage is superior to refrigerated storage for PDL cells. Trehalose has promising results as a storage media alone or in combination with HBSS or cell culture media.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Tooth avulsion is defined as a complete displacement of a tooth from its alveolar socket due to a traumatic injury (2). The management of this issue is both time and technique sensitive. The trauma that leads to a tooth avulsion causes damage to the cementum, periodontal ligament, alveolar bone, neurovascular supply to the pulp, and the surrounding gingiva (3).

Tooth replantation with monitoring follow-up visits after a traumatic avulsion is recommended by the American Association of Endodontists (4), American Academy of Pediatric Dentistry (2) and the International Association of Dental Traumatology (5). The goal of replantation is to have adequate healing of all damaged tissues. This is a complex healing process and relies primarily on time and the storage of the tissue prior to replantation (1, 5, 6).

The most significant and prevalent complication after the replantation of the avulsed tooth is external root resorption, predominantly replacement resorption and ankylosis (7-11). Defects in the PDL/cementum interface smaller than $4mm^2$ show more complete healing or transient ankylosis which is later resorbed and repaired. In contrast, defects larger than $4mm^2$ are more likely to develop permanent ankylosis (12).

Cell viability at the time of replantation is of utmost importance for proper repair of the damaged tissues (1, 6). PDL cell viability is a critical prognostic factor for replantation of avulsed teeth (13). In order to maintain cell viability, an avulsed tooth needs to be stored in an appropriate media to avoid desiccation. When an avulsed tooth is exposed to air and kept in a dry condition the PDL cells will become necrotic and result in a severe inflammatory response when replanted (14).

Hank's Balanced Salt Solution (HBSS) is currently the storage media that is recommended by the AAE, AAPD, and IADT for an avulsed tooth. HBSS is a sterile solution and contains essential nutrients for cell viability including: sodium chloride, D-glucose, potassium chloride, sodium bicarbonate, potassium phosphate, calcium chloride, magnesium chloride, and magnesium sulfate (15). EMT Toothsaver (Dentosafe) and Save-A-Tooth are two commercially available storage media for avulsed teeth. An ideal storage media will preserve cell viability of PDL cells and also be inexpensive and easily attainable by the public when necessary for tooth storage.

The purpose of this study was to compare current storage media and also investigate trehalose as another option for maintaining cell viability of human periodontal ligament fibroblasts for extended periods of time. The characteristic that makes trehalose of interest in this area of research is that it is found in many organisms which are capable of surviving either freezing or dehydration (16-18).

LITERATURE REVIEW

Dental Trauma/Avulsion

Tooth avulsion is defined as the complete displacement of a tooth from its alveolar socket due to trauma (2). Trauma occurs most commonly in children ages 7-12 and is usually caused by falls and accidents near school or home (19). Trauma to the anterior teeth occurs more commonly than posterior teeth and maxillary teeth more commonly than mandibular teeth (20). Avulsion injuries have been shown to make up 0.5-3% of all dental injuries (3, 21). Although it is not the most common dental injury, numerous studies show that it is one of the most serious dental injuries and prognosis relies heavily on the actions taken at the time of the accident (7, 22-24). Avulsion is a complex injury that affects multiple tissues. Damage includes disruption of the gingival epithelium, severance of the periodontal ligament, injury to cementum and alveolar bone, and severance of the dental pulp neurovasculature (25).

Healing of PDL/Cementum After Replantation

Numerous studies have found that minimizing damage to the PDL and cementum will lead to a greater ability for normal healing after replantation (12, 13, 26). The pattern of healing is complex and depends on the healing potential of each individual tissue and the rate at which that tissue will heal (27). Tooth avulsion may involve damage or injury to some or all of the periodontium including: gingival tissues, alveolar bone, cementum, and PDL. The healing process of the periodontium has been evaluated in multiple animal studies using histological analysis (13, 28, 29). These animal studies helped determine and support a chronology of normal healing after tooth avulsion. A

blood clot forms at the point of PDL tear or separation at 3 days post-replantation. Gingival healing is mostly complete at 7 days. The junctional epithelium has been re-established and gingival collagen fibers are reunited. Connective tissue also re-establishes the continuity of the previously separated PDL fibers. This connective tissue is composed of disorganized fibroblasts. At 1 month, the PDL will have a completely normal appearance but the connective tissue will not be fully mature histologically. At 4 months, the PDL fibers will be fully mature and functionally oriented (13, 28, 29). Healing of the PDL was analyzed in a recent study by Panzarini using histological and immunohistochemical analysis. This study, on replanted rat teeth, found that with immediate tooth replantation PDL regeneration may occur without causing external root resorption. It was also determined that healing of the PDL is mostly complete at 28 days and no remarkable changes are seen between 28 and 60 days post-replantation (30). A different study evaluated the biomechanical repair of the PDL after replantation by measuring the strength, stiffness, and toughness. The authors found that at 21 days after replantation the healing PDL had regained over 50% of its ability in all measures (31). Proper healing and repair of the cementum is also necessary for reattachment of PDL to the root surface. Damage to cementum can occur during avulsion and also during manipulation and replantation of the tooth. This damage leads to a repair process described by Andreasen and Hjorting-Hansen as surface resorption (7). This process is not actually a resorptive process, but instead represents the repair of the calcified tissue by recruitment of adjacent normal tissue (32). Lindskog et al evaluated the healing of cementum defects at a cellular level using scanning electron microscopy. The defects in cementum were initially invaded by macrophage-like cells which resorbed small areas of

dentin. As fibroblast-like cells began to invade the defect from the periphery the resorptive process was halted. After 6 weeks, the entire defect was filled with fibroblast-like cells, which were found to be producing new cementum over the defect. The cementoblast's ability to form a new cementum layer over the defect will allow for normal PDL attachment to the root surface (33). Repair of both cementum and PDL allow for normal attachment and function of the periodontium and prevent extensive resorption (34).

Resorption and Ankylosis

A study by Van Hassel et al evaluated replanted teeth of monkeys. Replanted teeth with intact PDL healed with normal PDL spaces in 9 of 21 teeth, whereas replanted teeth with removed PDL prior to replantation healed with a normal PDL in only 1 of 21 cases (26). Andreasen and Hjorting-Hansen defined resorption into three categories: surface resorption, replacement resorption, and inflammatory resorption (7). This study involved 110 avulsed teeth and evaluated the replanted teeth clinically and radiographically anywhere from 2 months to 13 years. The authors found that the replanted teeth could fall into three categories. The first category was a complete normal healing both radiographically and clinically. This process involved healing through surface resorption. Surface resorption has been defined as a localized area of damage to either the PDL or cementum during avulsion or replantation. As previously described, if this localized area is free from inflammation, it will undergo a normal healing process and proper attachment of PDL to cementum (7). The second category found by the authors was defined as replacement resorption. If damage to the PDL is more involved or completely removed during trauma, healing will occur from the alveolar side of the

socket. The space that was originally composed of PDL will be replaced by alveolar bone. This can lead to fusion of the alveolar bone directly to the root surface, resulting in ankylosis (35). In these such cases, replacement resorption occurs and eventually the entire root will be remodeled and replaced with bone. This process involves root resorption by osteoclasts followed by the deposition of alveolar bone instead of dentin (36, 37). Proper healing of the PDL has traditionally been determined using both radiographic and histologic examination. A loss of the normal radiolucent band surrounding the root structure (finding with a healthy PDL) is suggestive of ankylosis. Radiographic identification of ankylosis is not always possible with two dimensional radiography. Andersson et al found that only 11 of 31 ankylosed teeth were able to be correctly identified using two dimensional radiography (38). Clinical signs of ankylosis between the replanted tooth and the alveolar bone are arrested normal eruption, immobility of the tooth, and a high-pitch percussion sound (7). The third and final category that was described by Andreasen and Hjorting-Hansen was inflammatory resorption. For this resorption to occur there must be a combination of damage to the protective layer of cementum and the presence of inflammation (37). Inflammatory resorption can lead to complete resorption of a replanted tooth if left untreated. Radiographically, the teeth will exhibit radiolucent, bowl-shaped defects that penetrate into dentin (7).

The most prevalent complication after replantation of an avulsed tooth is replacement resorption followed by ankylosis (7-11). A study of 84 avulsed and replanted teeth with a 2 year follow-up found 40 teeth with replacement resorption and 22 teeth with inflammatory resorption (9). A larger clinical study of 400 replanted teeth

found 61% of cases to have signs of replacement resorption and 30% with a diagnosis of inflammatory resorption (8).

Importance of PDL Viability

Viable PDL cells are the most important prognostic factor in replanted teeth. An animal study found replanted teeth with necrotic PDL cells led to ankylosis, whereas vital PDL cells allowed for normal periodontal attachment after replantation (13). A study using replanted monkey incisors found that teeth that were stored in the vestibule of the animal for 90 minutes prior to replantation had normal PDL spaces radiographically and a normal PDL histologically at a 25 month follow up. However, teeth stored in a dry environment for 90 minutes showed radiographic and histologic signs of replacement resorption and ankylosis (39). The ability for a PDL to heal normally and return to its proper form and function is dependent on viable PDL cells at the time of replantation. Loe and Waerhaug studied replanted teeth in monkeys and dogs to determine the importance of PDL viability for regaining a normal attachment apparatus. All teeth that had the PDL removed showed signs of complete ankylosis within 30 days. Teeth that were air dried had localized areas of normal reattachment but mostly ankylosis. All teeth that were replanted immediately with a vital PDL regained the normal attachment apparatus (13). A study by Andreasen found that minimal extra-oral dry time, immediate replantation, and proper storage led to a better prognosis. They concluded that all of these factors allowed for more vital PDL cells and therefore increased the prognosis for normal PDL healing (8).

Multiple studies have pointed to reasons for abnormal responses of tissues after replantation including: major damage to PDL and or cementum during trauma, extended

dry time leading to desiccation of PDL cells, and unsuitable storage medium prior to replantation (1, 6, 40-42). It was found that damage to large areas of cementum during avulsion or handling during replantation can initiate the process of ankylosis. Lesions greater than 9 mm^2 resulted in permanent and complete ankylosis while lesions between 1 and 4 mm^2 had localized areas of ankylosis that resolved after 8 weeks (12). Soder performed an in vitro study using human and monkey extracted teeth to evaluate PDL cell viability after dry time. After 2 hours of dry time, no PDL cells were deemed viable (43). A few other animal studies confirmed the correlation between extra-oral dry time and devitalized PDL cells. After 60 minutes, there were very few vital PDL cells remaining and there were no vital PDL cells left after 120 minutes (1, 13). Andreasen and Kristerson performed a study where only a portion of the root was dried prior to replantation. Histological analysis revealed that areas of ankylosis occurred in the location corresponding to the dried areas (12). A study by Matsson et al using dogs found that dry time leads to significantly more ankylosis than a tooth stored in HBSS. Teeth stored in the solution for up to 60 minutes resulted in a negligible percentage of ankylosis, while teeth stored for 30 minutes dry time greatly increased the risk for ankylosis after replantation (45). This point is further proven in a study by Cvek using human subjects. Teeth that had been kept dry for over 60 minutes prior to replantation all showed signs of ankylosis. Teeth that had been dry for less than an hour but more than 15 minutes had signs of ankylosis in 60% of the cases. Teeth with dry time less than 15 minutes prior to replantation showed signs of ankylosis in 13% of the cases (6). In another study, the chance of ankylosis after 60 minutes of dry time in replanted dog teeth was found to be 89% (45). Chappuis and von Arx found in their study that 58% of

replanted teeth will regain a normal PDL attachment while the other 42% will undergo a form of resorption. Replacement resorption was the most common form occurring an average of 29% of the time. The incidence of replacement resorption was also found to increase with an increase in dry time. Replacement resorption occurred in 9.5% of replanted teeth after less than 15 minutes. A dry time between 15 and 60 minutes had an incidence of replacement resorption in 38.5% of cases. All replanted teeth with a dry time greater than 60 minutes showed signs of replacement resorption (10).

Immediate Replantation

Replanting an avulsed tooth immediately will allow for maximum PDL cell viability by reducing the extra-oral dry time. Immediate replantation has been described as the best possible treatment for an avulsed tooth by multiple authors (7, 8, 37). A study with dog teeth showed that immediately replanted teeth did not show signs of replacement resorption and ankylosis. A study of human replanted teeth determined that if an avulsed tooth was replanted immediately within 5 minutes of the incident there was a greater than 85% chance of normal healing (8). The same study also found that if the replantation was delayed to even as brief as 8 minutes the chance of normal healing could be less than 50%. An in vivo study examined the clonogenic capacity of the PDL progenitor cells. Clonogenic capacity is the ability of the vital progenitor cells to proliferate. Immediate replantation was found to be extremely important in this study because only 3% clonogenic capacity remained after 30 minutes of extra-oral dry time (46). Immediate replantation rarely occurs due to the nature of the accident, such as damage to the recipient site, more imminent life-threatening injuries, or simply lack of knowledge by the general population and medical personnel at the scene of the accident

about replantation procedures (47). If immediate replantation is not performed, it is important to store the tooth in a physiologic environment that allows for the greatest remainder of PDL cell viability.

Storage Media

The ability of the storage media to maintain PDL cell viability is one of the most important factors in the overall prognosis of avulsed teeth (48-50). A prospective clinical study found that delayed replantation after storage in a non-physiologic storage media led to a high chance of root resorption and eventual tooth loss (51). The ideal storage media is one that will maintain PDL cell viability, protect clonogenic capacity of progenitor cells, have little or no microbial contamination, be compatible with physiologic pH and osmolality, and be readily accessible to the public at a low cost (48-50, 52, 53).

Water has inadequate characteristics to be used as a storage medium for avulsed teeth due to its hypotonicity, non-physiological pH and osmolality, and possibility for bacterial contamination, which will lead to PDL cell lysis (53-55). Many studies have concluded that cells stored in water lead to rapid cell destruction (50, 56-58) and replanted teeth will show high levels of replacement resorption (1). It has been concluded that water is the least favorable storage media for an avulsed tooth and should be avoided (5, 46).

Alpha-Minimum Essential Media (MEM) cell culture medium contains L-glutamine, penicillin, streptomycin, Nistatin, bovine serum and nutrients for cell growth and proliferation (53). MEM has been shown to maintain the proliferative activity of PDL cells for extended periods of time (48-53 hours) and reduce the rate of resorption after replantation of an avulsed tooth (59-63). This solution has been used as a positive

control in avulsed tooth studies. Souza et al used MEM as a positive control and found that MEM showed the highest cell viability, although HBSS had similar results for the first 6 hours of storage (58). Multiple other studies have reported the efficacy of MEM cell culture media in preserving the viability of PDL cells and it has been suggested as a storage media prior to tooth replantation (53, 57, 64-66, 67).

Hank's Balanced Salt Solution (HBSS) is currently recommended by the AAE, AAPD, and IADT as the storage media of choice for avulsed teeth (2, 4, 5). This solution has been widely used as a reference in studies on tooth avulsion because of its ideal osmolality and pH for preservation of PDL cell viability. HBSS is a sterile solution that contains essential nutrients for cell preservation. These nutrients include sodium chloride, D-glucose, potassium chloride, sodium bicarbonate, potassium phosphate, calcium chloride, magnesium chloride and magnesium sulfate (15, 68). Its pH is between 7.2-7.4 (15, 56, 69, 70) and it has an osmolality of 275-284 mOsm/kg (56, 69-71) which are suitable conditions for cell growth (50, 54, 56, 64, 72-75). Hwang et al stored cultured human PDL cells in HBSS and found a 94% viability after 24 hours (56). Pillegi et al used extracted human teeth and observed 90% cell viability after storage in HBSS (74). This solution is strictly used in laboratory settings and has limited availability to the public or at the site of accidents (61), which is not ideal for immediate storage. Several studies have shown that HBSS is beneficial to PDL cells that were initially stored in a dry environment (73, 74, 76) or in saliva (77).

Save-A-Tooth was developed by Krasner as an emergency storage media. This product is an HBSS based media in a sealed container with a suspension net to help protect the tooth from damage as it is dropped into the system (78). The Save-A-Tooth

solution has a pH of 6.4-7.2 and osmolality of 275 mOsm/kg (65). Even though Save-A-Tooth is composed of HBSS it has shown inferior results to the original product (57, 67, 79).

EMT Toothsaver (Dentosafe) is a commercial product containing Special Cell Culture Medium (SCCM) including amino acids, vitamins and glucose (53). In vitro studies have shown SCCM to maintain viability of PDL cells comparable to HBSS for a time period of 24 hours (59, 80). It has also been demonstrated to maintain viability of PDL cells at room temperature for at least 48 hours (81). The media is marketed as Dentosafe in Europe. Since its distribution in Germany to many schools, the rate of functional healing after replantation of avulsed teeth has increased to 50% (81).

Trehalose Background

Trehalose (α,α-trehalose) is a disaccharide composed of two D-glucose molecules formed in a 1,1 linkage (16). Its glycosidic bond is not cleaved by α-glucosidase and it is a non-reducing sugar that is not easily hydrolyzed by acid (82). Although trehalose is a naturally occurring disaccharide, it has some unique physical and chemical characteristics that differentiate it from other more common sugars. Trehalose has a high degree of optical rotation and also a unique melting behavior. The initial melting point of trehalose is 97ºC, it re-solidifies at 130ºC when heat drives off the water, then the anhydrous trehalose melts at 203ºC. The physical and chemical properties of trehalose cause it to be an extremely stable disaccharide (16, 17).

The history of trehalose began in 1832 when H.A. Wiggers is given credit for discovering the sugar while studying solutions of the ergot of rye (83). In 1974, Elbein showed that trehalose was present in over 80 different species of plants, algae, fungi,

yeasts, bacteria, insects, and other invertebrates. Trehalose has not been identified in higher species such as mammals, however, the enzyme that cleaves trehalose (trehalase) has been found in the small intestines and other organs of various species (17). The role of trehalose in different organisms seems to be multifactorial. In lower organisms, it is used as an energy source during development, while in insects, it may be used as an energy source for flight. In mycobacteria, it has been shown to be incorporated into structural components (17).

The sawfly larva, which contains high levels of trehalose, can survive temperatures of -40^{o}C (86). The resurrection plant is composed of about 12.5% dry weight trehalose. This plant has the ability to become completely dried out, then upon rehydration, it will return to absolutely normal activity (85). A study of yeast Saccharomyces cerevisiae found that a chemical property of trehalose contained within the organism was directly responsible for the desiccation tolerance (86). Additional studies have confirmed that trehalose plays a significant role in anhydrobiosis, which is the ability of cells to withstand prolonged periods of desiccation (87-89).

The mechanism of action for its protective activity has been described in three categories: water replacement, glass transformation, and chemical stability (90). Hydration with water normally plays a role in stabilization of biologic macromolecules through hydrogen bonding. During periods of dehydration these molecules lose their stability and denature. It has been proposed that trehalose interacts with the polar groups in biomolecules to stabilize the organism and its structures during times of dehydration (90). Rehydration can then allow for normal cellular activity without major damage that could normally follow periods of desiccation (89). Glass transformation is a process of

trehalose sugar in solution forming a non-hygroscopic glass that is stable at high temperatures and periods of complete desiccation (91). This property allows trehalose glass to maintain structure and keep biomolecules in their native form and capable of normal function following rehydration. Finally, trehalose is one of the most chemically stable sugars and is highly resistant to hydrolysis (16). The physical and chemical properties of trehalose make it a unique disaccharide capable of maintaining cellular structure and function during times of temperature and dehydration stresses.

Trehalose is synthesized primarily by anhydrobiotes, which mostly include bacteria, yeasts, nematodes, and some crustaceans and insects (91, 92). Trehalose has been recently used in studies for cryopreservation and lyopreservation of mammalian cells due to its protective abilities during times of stress in anhydrobiotes. Mammalian cells do not synthesize trehalose nor do they have the transports in their membranes to import trehalose, which has been a drawback of its use in preservation of mammalian cells. An engineered form of trehalose has been proposed to help increase the permeability of trehalose into mammalian cells for better biopreservation (93). Both intra- and extra-cellular trehalose have been shown to have protective abilities on cells from stresses such as cold and desiccation (94-100). The best results in protection of cells, however, was observed when it is present on both sides of the cell membrane (94-100).

A study of cryopreservation of mouse oocytes found that extracellular trehalose alone afforded some protection of cells from subzero temperatures, but diminished as temperatures dropped below -30^{o}C (101). Lyopreservation, the storage of cells in a desiccated state at ambient temperature, is a simple and cost effective method of

biopreservation. Abazari et al evaluated desiccated nucleated mammalian cells stored with both intra- and extra-cellular trehalose. Extra-cellular trehalose film exhibited an amorphous state with a water to trehalose ratio of 2:1 (102). Another study stored human hematopoietic stem and progenitor cells at 25^{o}C for up to 4 weeks after lyophilization. Trehalose was loaded into these cells, they were freeze-dried and then stored at ambient temperature. It was found that there was high retention of differentiation and clonogenic potential (103).

MATERIALS AND METHODS

Institutional Review Board Approval

This in vitro study using cultured periodontal ligament fibroblasts from human extracted teeth was approved by the Marquette University Graduate School. An exemption status was granted by the Institutional Review Board of Marquette University.

Cell Culture of Human Periodontal Ligament Fibroblasts

All cell culture procedures were completed under a laminar flow tissue culture hood with proper aseptic techniques. The human periodontal ligament fibroblasts (hPDLF) were obtained from the Department of Developmental Sciences at Marquette University. The original cells were purchased from ScienCell Research Laboratories and had been frozen for preservation and storage.

The frozen cells were thawed and incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air for two days. The cells were fed with Eagle's Alpha-Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml) (PS), 1% amphotericin (250 µg/ml) (A) and 5mM L-Glutamine (L) (EMEM-FBS-PS-A-L). After cells reached 90% confluence they were observed under a microscope to confirm their fibroblast-like appearance. At this point, the cells appeared thin and elongated as expected by fibroblasts and firmly attached to the cell culture flask (Figure 1). The cells were sub-cultured using the following steps in order to obtain the necessary number of cells for the experiment. The cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) and TrypLE^{TM} Express Enzyme was added to the flask. The cell culture flask was rocked

gently and then incubated at 37°C in 5% CO_2 and 95% air for 5 minutes. The flask was observed under the microscope to confirm the cells had rounded and detached from the surface of the cell culture flask (Figure 2). TrypLE™ Express Enzyme was neutralized by adding EMEM-FBS-PS-A-L that was pre-warmed to 37°C. The cell suspension was then transferred to a 15mL conical tube and centrifuged at 100xg for 5 minutes. The cell pellet was isolated and re-suspended in the 37°C EMEM-FBS-PS-A-L. The suspension was transferred into a new 75 cm^2 cell culture flask and incubated at 37°C in 5% CO_2 and 95% air. The cells were incubated and re-fed with EMEM-FBS-PS-A-L every 2 days until the cells reached 90% confluence. After one passage, the cell count was sufficient for the experiment.

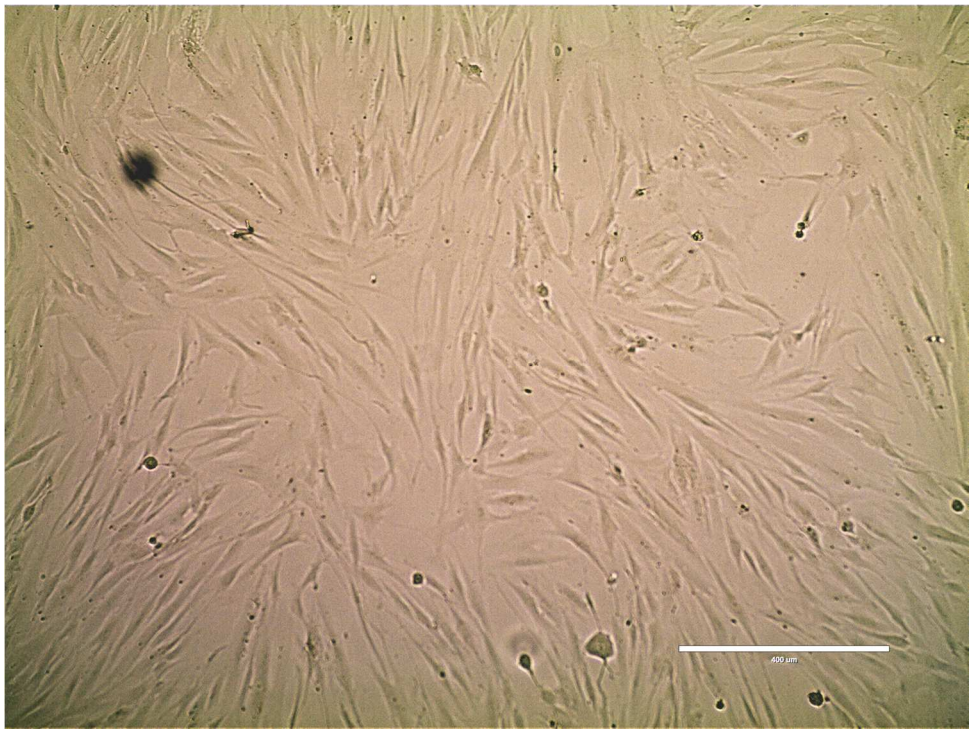

Figure 1. Visualization of 90% confluent fibroblast-like human periodontal ligament cells under magnification.

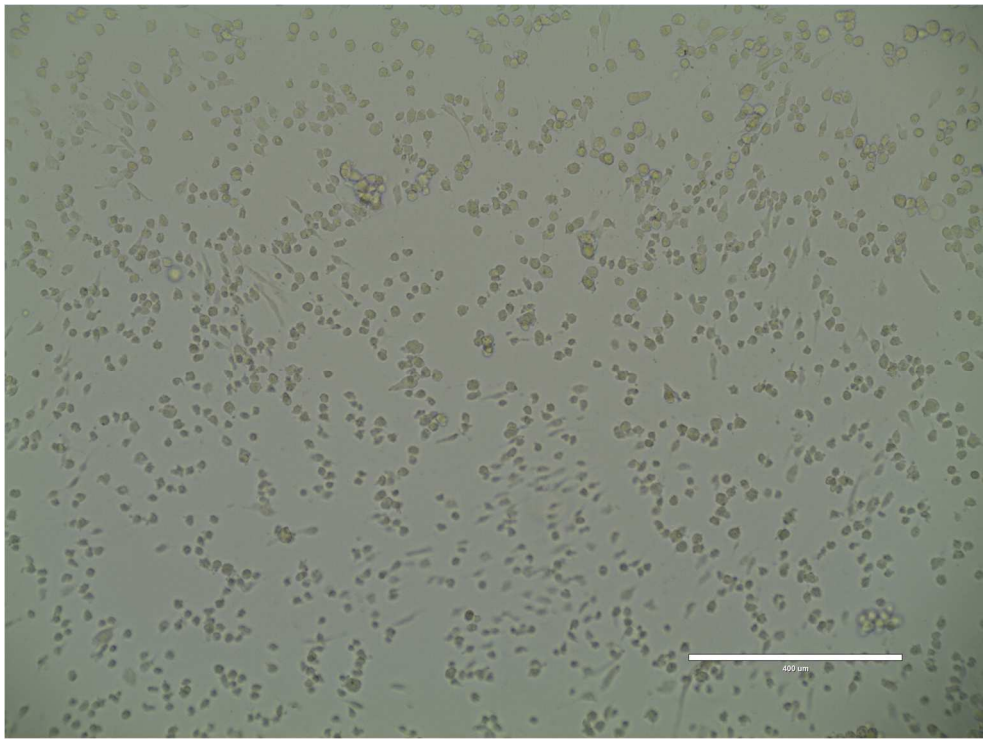

Figure 2. Visualization of rounded hPDLF cells after trypsinization under magnification.

PDL Cell Distribution

The cells were detached from the cell culture flask using TrypLE™ Express Enzyme as described previously. After the cells were centrifuged and re-suspended in EMEM-FBS-PS-A-L they were counted under magnification by trypan blue exclusion staining. The cell suspension was agitated to evenly distribute cells throughout the flask. Cells were then seeded on 96-well culture plates in identical volumes. The calculated cell number per well was estimated to be 5000 cells. The cell culture plates were incubated at 37ºC in 5% CO_2 and 95% air for 24 hours to allow for attachment of hPDLF to the culture plate surface.

Storage Media

The current commercial products, EMT Toothsaver and Save-A-Tooth, were stored and used in accordance with manufacturer's instructions (Figure 3). Sterile HBSS with calcium and magnesium was stored at room temperature (22ºC). A solution of 300 mM trehalose (T300) (Figure 4) with distilled water and a cell culture media (DMEM+10%FBS+6mM L-Glutamine+2mM Sodium Pyruvate) were produced as experimental storage media. Combinations were also created as experimental groups including: HBSS with 50 mM trehalose (HBSS/T50), HBSS with 150 mM trehalose (HBSS/T150), cell culture media with 50 mM trehalose (media/T50), and cell culture media with 150 mM trehalose (media/T150). Cell culture media (DMEM+10%FBS+6mM L-Glutamine+2mM Sodium Pyruvate) incubated at 37ºC in 5% CO_2 and 95% air was used as a positive control and distilled water at room temperature was used as a negative control.

Figure 3. EMT Toothsaver (Dentosafe) and Save-A-Tooth storage media commercial products.

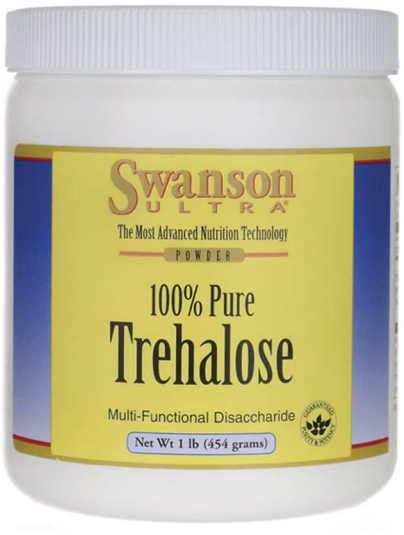

Figure 4. Trehalose powder used to create storage media solution with distilled water.

Addition of Storage Media to Cells

After 24 hours of incubation and confirmation of cell attachment to the 96-well plate, the cell culture media was removed from each well. Each of the 10 different types of storage media (Water, HBSS, HBSS/T50, HBSS/T150, media, media/T50, media/T150, T300, SAT, EMT) were place into a well in the amount of 100µL. The positive control had 100µL of media added and was placed in the incubator. One group of plates was placed in a refrigeration unit at 4^{o}C and the other group of plates was left at room temperature (22^{o}C). Cells were left in storage media for time points of 1, 2, 6, 12, 24, and 48 hours prior to analysis via MTT assay.

Measuring Cell Viability

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was used in this study to detect cell viability. The MTT assay has been widely used in academic labs for this purpose. Viable cells with active

metabolism will convert MTT into a purple colored formazan product (104). The exact mechanism is not completely understood but likely involves reaction with NAD(P)H and mitochondrial enzymes (105). The purple colored formazan has an absorbance maximum near 570nm (104). When cells die, they lose their ability to convert MTT into formazan, therefore the formation of color serves as a useful indicator of only the viable cells. After cells had been stored for the desired time, the following protocol was followed for the MTT assay. The MTT was dissolved in Dulbecco's Phosphate Buffered Saline to 5 mg/mL and stored at 4^{o}C. This storage container was wrapped in foil to protect it from light. Then 10µL of MTT solution was added to each well and incubated for 3 hours at 37^{o}C. After incubation, the solution and storage media was removed from each well. Dimethyl sulfoxide (DMSO) was used as the solubilization solution in order to dissolve the formazan crystals. 100µL of DMSO was added and mixed with the pipet to ensure complete solubilization (Figure 5). The plates were read at an absorbance of 570nm on a Synergy HTX multi-mode plate reader.

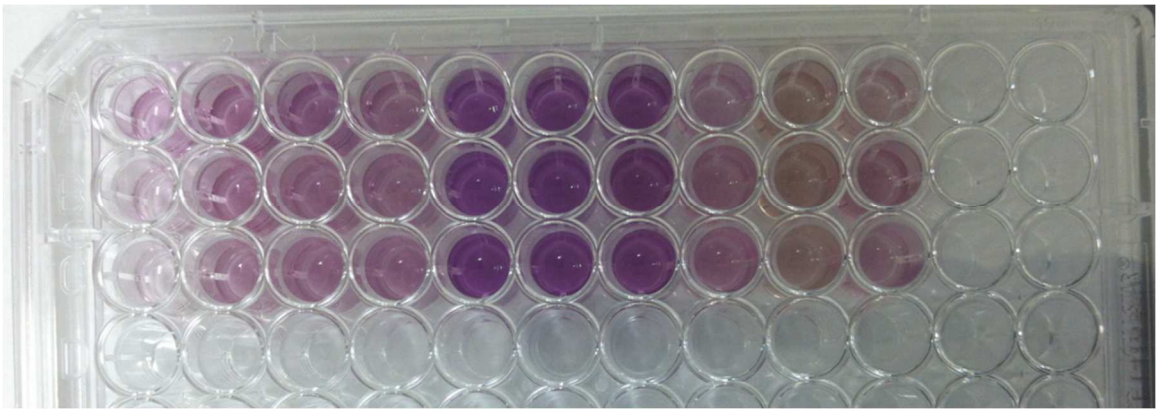

Figure 5. Image of 96-well plate containing all 10 storage media after MTT assay was complete.

Statistical Analysis

The cell absorbance values from the MTT assay were analyzed using a Univariate General Linear Model (GLM), which tested the effects of time, the medium used, and their interaction. Tukey's HSD was used for post hoc analysis. These analyses were performed separately for the 22°C and 4°C trays. Relationships were considered significant for p-values < 0.05.

RESULTS

All storage media were compared using the methodology described to determine the overall performance of the media over a 48 hour time period at room temperature and refrigerated temperature. The absorbance values for all media have been plotted over the 48 hour time period. Because of the large number (10) of storage media used, graphs were separated for visual readability. The graphs were separated based on performance in comparison to HBSS, since HBSS is currently recommended by the AAE, AAPD, and IADT as the storage media of choice for avulsed teeth (2, 4, 5). HBSS and the media that performed better were graphed separately from HBSS and the media that performed worse, according to the GLM results (Figures 6-9).

Storage Media at Room Temperature

The positive control of cell media in an incubator performed significantly better than all other storage media, as expected. The mediaT150, mediaT50, and media performed the highest out of all experimental groups but only mediaT150 was significantly better than the next best storage media, HBSS. HBSS, HBSST50 and HBSST150 were not significantly different from each other but were all significantly better than T300, SAT, EMT, and water. T300 performed better than SAT, EMT and water but was only significantly better than water. SAT and EMT were not significantly different from water (Table 1).

Table 1. Observed mean values calculated from Tukey's HSD post hoc to compare overall performance of each media at room temperature (22°C). Storage media values that share the same group are not significantly different from each other. Different groups are significantly different from one another.

Room Temp. Tukey's HSD		Group					
Medium	n	X	A	B	C	D	E
Incub. Media	18	0.74622					
Media T150	18		0.53800				
Media T50	18		0.51789	0.51789			
Media	18		0.49511	0.49511			
HBSS	21			0.45552	0.45552		
HBSS T50	18				0.40128		
HBSS T150	18				0.31989		
T300	18					0.27333	
SAT	18					0.20106	0.20106
EMT	18					0.19961	0.19961
Water	21						0.18619
Significance		1.000	0.786	0.260	0.234	0.087	1.000

The cell media groups (mediaT150, mediaT50, media) had higher absorbance values recorded up to the 12 hour time point. At the 24 hour time period, the cell media groups had significant decreases in absorbance. HBSS, HBSST50, and HBSST150 had a lower performance than the cell media group for the first 12 hours but maintained a more stable absorbance value through the 24 hour time period. At the 48 hour time point, the HBSS group values had a significant decrease. T300 had a lower performance compared to the HBSS group throughout the 48 hours. However, T300 did follow a similar pattern, in that it remained stable through 24 hours and did not have a significant drop in absorbance values until 48 hours. SAT had similar performance to T300 for the first 6 hours but had a significant decrease in absorbance values at the 12 hour time point. EMT and water both had a significant drop in absorbance in the first hour, however EMT did

have a significant resurgence in absorbance value at the 12 hour time period where it was comparable to T300 (Figure 6, 7 and Table 2).

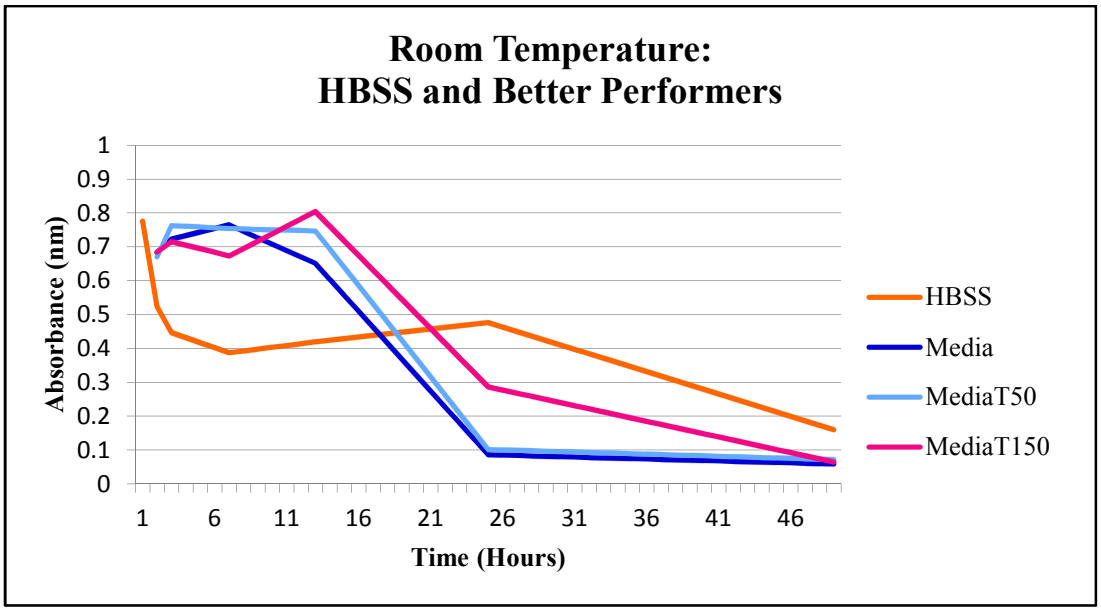

Figure 6. Absorbance values recorded over 48 hours at room temperature (22ºC) for HBSS and those storage media that performed better than HBSS.

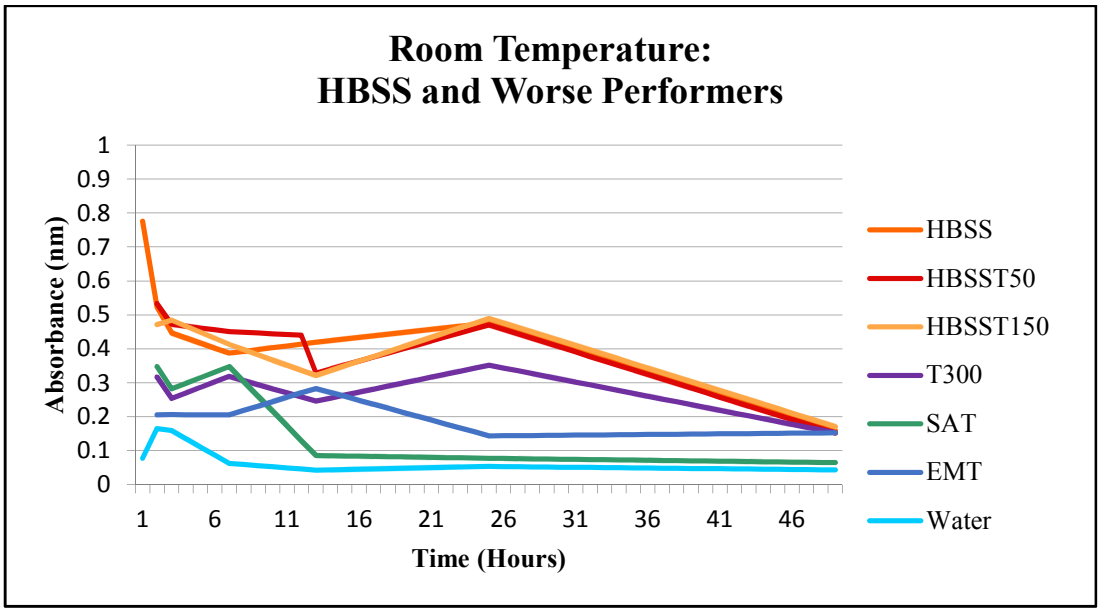

Figure 7. Absorbance values recorded over 48 hours at room temperature (22ºC) for HBSS and those storage media that performed worse than HBSS.

Table 2. Storage media comparisons at room temperature (22°C) including time at which absorbance decreases or resurges and the performance groupings previously discussed.

Medium	Significant Decrease (Hour Time Point)	Significant Resurgence (Hour Time Point)	Group (Table 1)
MediaT150	24		A
MediaT50	24		A,B
Media	24		A,B
HBSS	48		B,C
HBSST50	48		C
HBSST150	48		C
T300	48		D
SAT	12		D,E
EMT	1	12	D,E
Water	1		E

Storage Media at Refrigerated Temperature

At refrigerated temperature, the best performing storage media included mediaT150, HBSST150, mediaT50, media, and HBSST50. This group of storage media were not significantly different from each other, but only mediaT150, HBSST150, and mediaT50 were significantly better than all other storage media tested. The next highest performing group included T300 and HBSS, which were not significantly different from HBSST50 and media. HBSS was significantly better than SAT, whereas T300 had no significant difference from SAT. SAT was not significantly different from EMT but was

significantly better than water. EMT was the worst performing experimental group and had no significant difference from water (Table 3).

Table 3. Observed mean values calculated from Tukey's HSD post hoc to compare overall performance of each media at refrigerated temperature (4ºC). Storage media values that share the same group are not significantly different from each other. Different groups are significantly different from one another.

Fridge Temp. Tukey's HSD		Group				
Medium	n	A	B	C	D	E
Media T150	18	0.38767				
HBSS T150	18	0.35928				
Media T50	18	0.35539				
Media	18	0.33439	0.33439			
HBSS T50	18	0.33439	0.33439			
HBSS	18		0.27872			
T300	18		0.27550	0.27550		
SAT	18			0.21222	0.21222	
EMT	18				0.14989	0.14989
Water	18					0.08917
Significance		0.183	0.093	0.051	0.059	0.073

At refrigerated temperatures, the cell media groups (mediaT150, mediaT50, media) had a better performance than all other media for the first 6 hours and all showed a significant decrease at 12 hours. MediaT150 showed the smallest decrease at this time point compared to the other media groups. HBSST150 and HBSST50 also performed slightly better than HBSS alone and showed a similar decrease at the 12 hour time point. The HBSS group, especially HBSST150, showed a more consistent absorbance value from the 12 to 48 hour time period. SAT performed similar to HBSS in the first 2 hours but had a significant decrease at the 6 hour time point. EMT and water both showed significant drops in absorbance value in the first hour. T300 had a slightly lower performance than HBSS in the first 6 hours but did not have a significant decrease

throughout the 48 hour time period. T300 had similar absorbance values as HBSST150 from the 12 to 48 hour time period (Figures 8, 9 and Table 4).

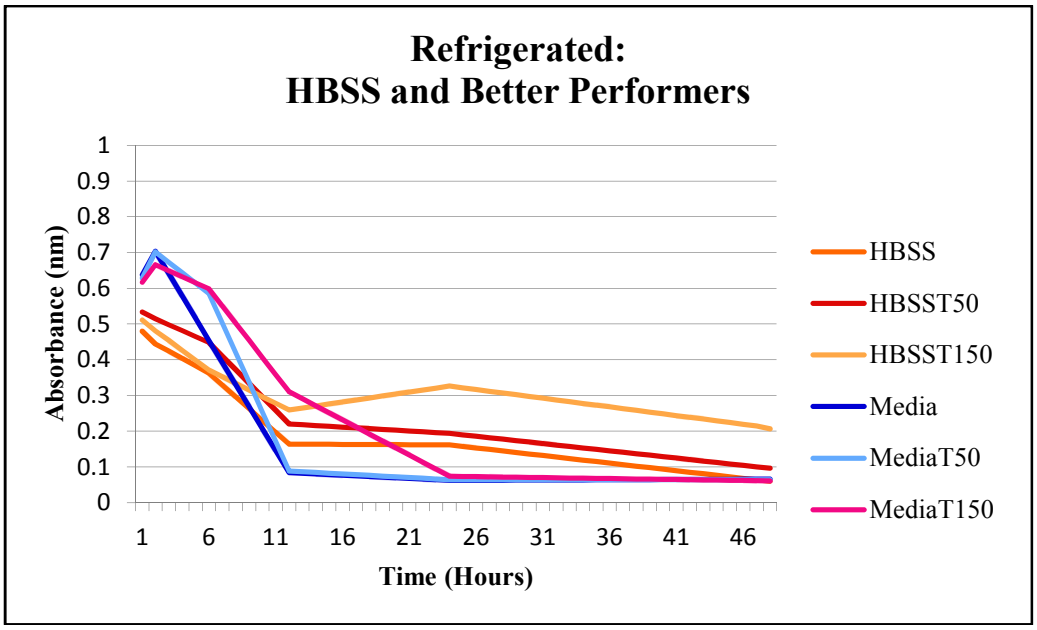

Figure 8. Absorbance values recorded over 48 hours at refrigerated temperature (4ºC) for HBSS and those storage media that performed better than HBSS.

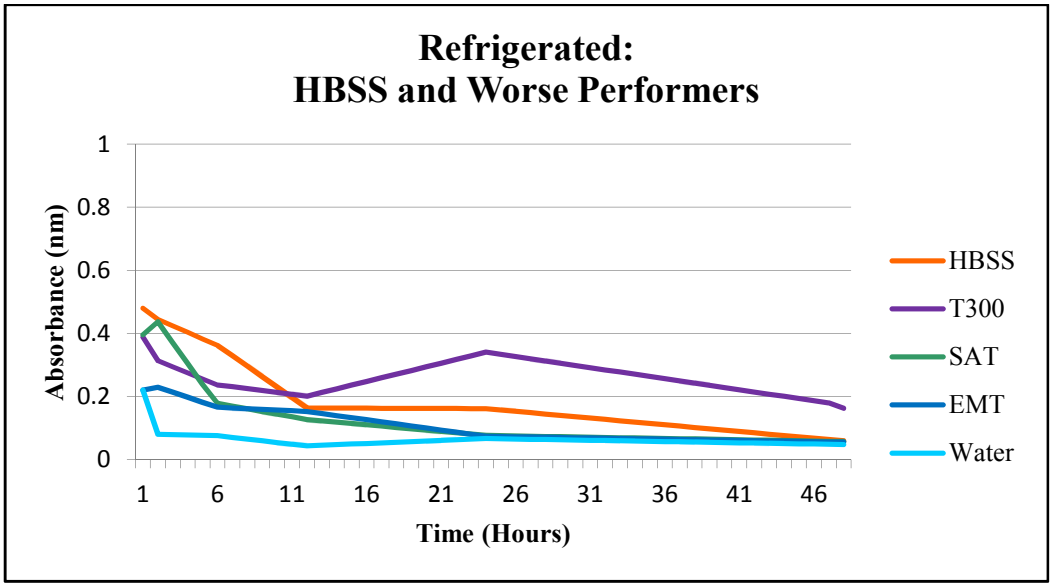

Figure 9. Absorbance values recorded over 48 hours at refrigerated temperature (4ºC) for HBSS and those storage media that performed worse than HBSS.

Table 4. Storage media comparisons at refrigerated temperature (4ºC) including time at which absorbance decreases or resurges and the performance groupings previously discussed.

Medium	Significant Decrease (Hours)	Significant Resurgence (Hours)	Group (Table 2)
MediaT150	12		A
HBSST150	12		A
MediaT50	12		A
Media	12		A,B
HBSST50	12		A,B
HBSS	12		B
T300	NSD		B,C
SAT	6		C,D
EMT	1		D,E
Water	1		E

Storage Temperature Comparison

All storage media were tested at both room temperature and at refrigerated temperature as well as a positive control in an incubator at 37ºC. Cell culture media was used to form a comparison between all three temperatures. Absorbance values were the highest in the incubator group, followed by room temperature, and the lowest values were found in the refrigerated temperature. The incubator did not have a significant decrease, but its lowest absorbance value was found at 48 hours. The room temperature group had a significant decrease at 24 hours. The refrigerated temperature group had a significant decrease at 12 hours (Figure 10).

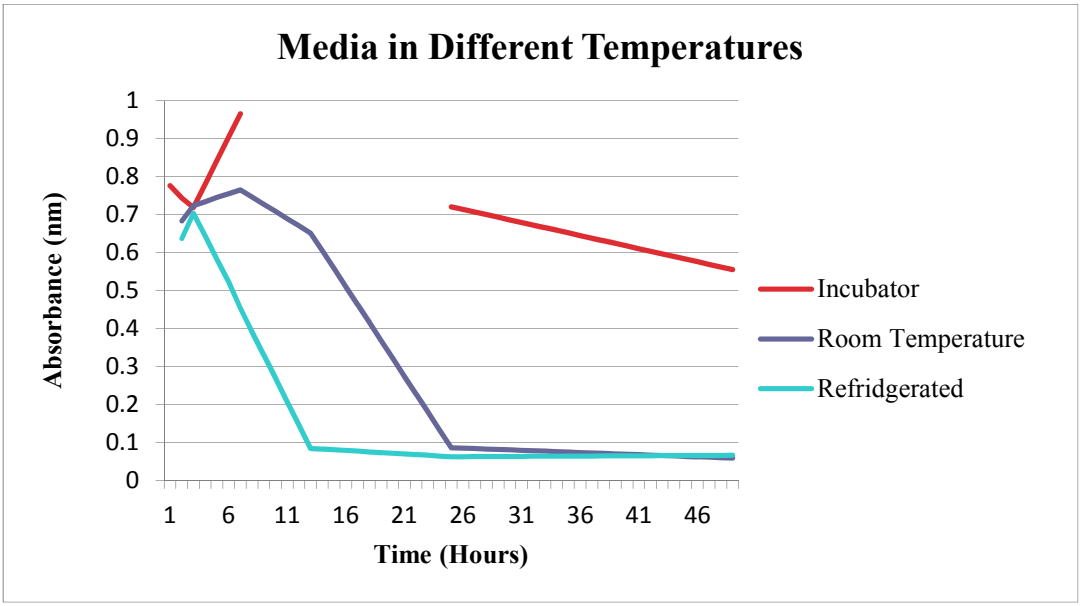

Figure 10. Absorbance values of cell culture media at positive control temperature (37ºC), room temperature (22ºC), and refrigerated temperature (4ºC) over the 48 hour time period (12 hour time point was not collected with positive control due to incubator being inaccessible at this time during experiment).

DISCUSSION

At room temperature, it was determined that the current commercially available storage media (Save-A-Tooth and EMT Toothsaver) did not perform significantly better than water in preserving cell viability. Water, EMT, and SAT had the quickest decrease in cell viability at 1, 1, and 12 hours respectively. Water is known to cause cell lysis and is contraindicated for storage of an avulsed tooth. Based on the findings from this study, EMT and SAT are not recommended as storage media because they did not perform significantly better than water and cell viability appears to decline rapidly after storage.

Trehalose did perform better than water but did not maintain cell viability as well as HBSS. T300 and the HBSS groups did not have a significant decrease in viability until the 48 hour time point. Trehalose does maintain cell viability over 24 hours but does not perform as well as HBSS and should not be recommended to be used over HBSS at this time. The combination of trehalose with HBSS did not improve the performance over HBSS alone.

The mediaT150 mixture had the best performance and was significantly better than HBSS. The other cell culture media (mediaT50 and media) were not significantly different from HBSS. MediaT150, mediaT50, and media all had significant decreases in viability at the 24 hour time point. MediaT150 shows promise as a storage media especially over the first 12 hours. This mixture out performed all other storage media in this study. If a tooth must be stored for more than 24 hours, HBSS may be a better recommendation due to the ability to maintain a higher viability until 48 hours.

At the refrigerated temperature, there were similar findings to room temperature. EMT did not perform significantly different from water and both had decreases in cell

viability immediately. Based on these findings, EMT is not recommended as a storage media at this temperature. SAT showed better results than water but still had a significant decrease in viability at 6 hours.

Trehalose performed as well as HBSS at the refrigerated temperature, which can be attributed to the ability of trehalose to help resist cellular damage at low temperatures. Treahlose did not show a significant decrease in viability throughout the 48 hour time period. The stability of absorbance values over the entire 48 hour time period helps verify that trehalose does have an ability to maintain cell viability at extended periods of low temperature.

MediaT150, HBSST150, and mediaT50 had the best performance at refrigerated temperature and were significantly better than HBSS. All of these groups had significant decreases at 12 hours. The trehalose mixtures with HBSS and media may have performed superior due to the protective abilities of trehalose at this temperature. Overall, the storage media maintained a higher cell viability and for longer periods of time at room temperature as compared to refrigerated temperature.

Performance Reasoning, Study Limitations, and Errors

Trehalose performed better than the commercial products overall, but did not maintain cell viability better than HBSS or cell culture media. T300 was the only storage media to maintain cell viability without a significant decrease at the refrigerated temperature, while mediaT150 outperformed all other storage media at room temperature. This could be attributed to trehalose's previously discussed ability to stabilize and protect cells from freezing temperatures. The mediocre overall performance could be attributed to pure trehalose's inability to transport across mammalian cell membranes. Recent

studies have attempted to identify ways of permitting trehalose to transport into mammalian cell cytoplasm in order to have increased cellular preservation characteristics. A recent study by Abazari has found that by enhancing the small molecule lipophilicity of trehalose it will enhance the transport across the cell membrane. This is done by replacing the hydrophilic hydroxyl groups with lipophilic acetyl groups. Trehalose hexaacetate was found to be the best structure for cell permeability and showed up to 10 fold more intra-cellular trehalose compared to extra-cellular trehalose (93). Trehalose has capabilities of maintaining viable cells through extremely low temperatures and desiccation in plants, algae, fungi, yeasts, bacteria, insects, and other invertebrates. In this study, trehalose did not perform as well as expected. This is most likely due to the lack of intra-cellular trehalose in the PDL fibroblasts. A follow-up study using trehalose hexaacetate would be ideal to determine if intra- and extra-cellular trehalose would lead to better cell viability preservation. Due to the cost of engineered trehalose this may not be applicable for a commercial tooth storage media. Additional information about the ability of trehalose and trehalose hexaacetate to preserve cells could be especially important in organ and tissue preservation.

Cell culture media performed better than HBSS, especially over the first 12 hour time period at room temperature. MediaT150 was found to be significantly better in overall performance compared to HBSS. Cell culture media most likely performed well over the first 12 hours due to providing cells with nutrients necessary for survival. After the nutrient resources are depleted, the cell viability decreased. MediaT150 had the best performance overall due to the high cell viability in the first 12 hours and the decrease in cell viability was not as dramatic over the remaining 36 hours. The combination of

nutrient sources from the cell culture media and the protective effects of trehalose formed a significantly improved tooth storage media compared to currently available commercial products.

EMT and SAT did not perform well in this study in regards to maintaining cell viability over a reasonable amount of time. This finding conflicts with a previous study which found EMT to perform similarly to HBSS over a 48 hour time period (106). This contradictory finding could be due to the different assay techniques used when measuring cell viability. In this study, the MTT assay was used as described earlier. This assay is designed to identify cells that are metabolically active and capable of converting MTT into a purple colored formazan product. This mechanism is most likely carried out via NAD(P)H and other mitochondrial enzymes. As long as a cell still has a functioning mitochondria and metabolism, it will be considered viable by this test. In the previous study, an ATP assay was performed to measure viability. This assay method measures ATP using firefly luciferase. This technique uses cell membrane integrity to define a viable cell. If the cell membrane loses its integrity the cell will no longer produce ATP and the endogenous ATPases will rapidly deplete any remaining ATP (104). This technique does not require an incubation step prior to reading, whereas the MTT assay requires at least an additional 1-3 hours of incubation for the formation of formazan product.

There are a number of assay techniques that focus on different cell characteristics to determine viability. This leads to differing results among studies, based on the status of different cellular components. These assays are designed to give a good estimate of viable cells at the time of measurement. One cannot assume that all of these cells are

completely devoid of damage and would continue to function and proliferate when placed back in to their physiologic environment.

There are also a few features of this experiment that may have led to possible errors in cell viability measurements. The MTT assay requires 3 hours to incubate prior to measuring absorbance. This time in the incubator was not included in the time point measurements. Therefore, after the stated length of time at the tested temperature, cells were placed in the incubator for an additional 3 hours prior to measurement. It is possible that the addition of MTT with a storage media and incubation for 3 hours could have effects on cell viability. A separate study was run in which all storage media were removed prior to addition of the MTT and incubation. The separate study found EMT to perform significantly better. It is hypothesized that EMT may be incompatible with MTT and the interaction between the two during the assay led to a decrease in overall performance.

Another possible source of error could be attributed to the difference in color of the storage media. The MTT assay measures a color pigment to determine cell viability. Some of the storage media used in the experiment were colorless (trehalose and SAT) while others (cell culture media, HBSS, and EMT) had a pink color similar to that of the MTT assay. To limit the possibility of storage media pigment altering the absorbance values, the storage media and MTT were removed from each well prior to the addition of DMSO. If some of the media was left in the well prior to the addition of the DMSO it is possible that this could have led to a deeper color change and increased the absorbance reading.

It is important to also understand that this was an in vitro study using PDL fibroblast cells attached to cell culture plates. This information can not be directly translated to the clinical setting. However, these studies are necessary in order to compare storage media and help develop new possibilities. After a storage media has been proven successful in vitro, follow up studies can evaluate it on extracted teeth with PDL cells that are intact with root cementum. The latter studies would be more reliable for translation of results to a clinical setting. This still does not take in to account the replantation of a tooth back in to the alveolar socket. Many more variables are involved in a true clinical setting. Cell viability, healing, and function after replantation can not accurately be determined through the current in vitro experiments. The shelf life, storage, and ease of use of different media are also important for the public. An ideal tooth storage media is easily accessible to the public, inexpensive, easy to store, long lasting, and capable of preserving a large percentage of cell viability for an extended period of time.

CONCLUSIONS

Cell culture media in combination with 150mM trehalose had a significantly better performance than HBSS as a storage media at room temperature, especially over the first 12 hours. Cell culture media with and without trehalose should be further evaluated as a potential storage media. HBSS with and without trehalose maintained higher cell viability from 24 to 48 hours and HBSS should continue to be a recommended storage media for avulsed teeth until superior results are reproducible in vivo. The current commercially available storage media did not perform well. Based on the results of this study, SAT and EMT should not be the recommended as an ideal storage media. Room temperature storage is superior to refrigerated storage for PDL cells. Trehalose has promising results as a storage media alone or in combination with HBSS and cell culture media. Additional research should be conducted in this area for the development of more effective commercially available storage media.

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