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Survival of human periodontal ligament cells in media proposed for transport of avulsed teeth

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Abstract – Many solutions have been examined as possible storage media for avulsed teeth. In this report, human periodontal ligament (PDL) cells were exposed for 1 h to culture medium, milk, Hanks Balanced Salt Solution (HBSS), Soft Wear, Opti Free, and Solo Care contact lens solutions, Gatorade®, and tap water, at room temperature and on ice. The number of viable cells was counted using the trypan blue exclusion technique, immediately after exposure (0 h) and at 24 and 48 h, to test the proliferative capacity of the cells after treatment. The results indicated that a significantly higher number of cells survived and proliferated when the exposures were performed at 0°C. Water had a detrimental effect on the cells, whereas culture medium and HBSS preserved significantly more viable cells than the other experimental solutions. Within the parameters of this study, it appears that HBSS is the optimal storage medium for avulsed teeth. Low-fat milk could serve as an alternative if ice is available. Contact lens solutions or Gatorade® on ice could serve as short-term (1 h) storage media if the other solutions are not readily available.

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According to the World Health Organization's (WHO) classification of dental injuries, avulsion (exarticulation) is a complete displacement of a tooth from its alveolar socket (1). Avulsion is a complex injury that affects multiple tissues (2, 3). It accounts for up to 16% of all traumatic injuries in the permanent dentition (4–9), and for 7–21% of injuries in the primary dentition (4, 10).

The damage to the attachment apparatus during an avulsion injury is unavoidable, but maintaining the viability of the periodontal ligament (PDL) that is attached to the avulsed tooth is critical. One of the most important factors determining the prognosis for the tooth is the length of extra-alveolar time (3, 11, 12). Ideally, the tooth should be replanted immediately after the injury in an effort to preserve the viability of the PDL cells, and so to optimize healing and minimize root resorption (13).

Although immediate replantation has been shown to have the best prognosis (12, 14–18), unfortunately, this rarely occurs. When immediate replantation of an exarticulated tooth is not possible, the storage conditions should be designed to maximize preservation of the PDL during transportation to the dental office (19).

Cvek et al. (20) demonstrated that 13% of teeth kept in a dry state for 15 min, 40% of those kept in a dry state for 20–40 min, and 100% of those stored dry for >60 min showed signs of ankylosis. Therefore, it is most important to prevent the PDL cells from drying. Dry extra-oral time causes irreversible damage to the PDL cells, which upon replantation elicit an inflammatory response over a diffused area on the root surface, resulting in ankylosis and ultimately in loss of the tooth (3).

If immediate replantation is not feasible, Axhausen (21) suggested keeping the tooth under the patient's

tongue until it is brought to the dentist. However, milk has been proven to be a more suitable storage medium for avulsed teeth, mainly because of its physiologic osmolality, neutral pH, presence of essential nutrients, and lack of active toxic components (19, 22–26). In The American Association of Endodontists (AAE) (27) guidelines for the treatment of avulsed teeth, Hanks balanced salt solution (HBSS; Life Technologies, Grand Island, NY, USA) is listed as the storage medium of choice because of its ability to preserve the viability of the majority of the PDL cells for extended periods of time (19, 28–32). HBSS is non-toxic, pH balanced (7.2), and has an osmolality appropriate for cell growth. Hiltz & Trope (30) showed that HBSS was very effective in preserving the viability and morphology of lip fibroblasts for up to 72 h. Harkacz et al. (33) and Olson et al. (34) tested the efficacy of Gatorade® (Thirst Quencher – Starfruit, Gatorade, Chicago, IL) as a transport medium for PDL cells. At room or body temperature, Gatorade® was found to be inferior to the negative controls (dry or tap water) when comparing the numbers of remaining viable cells after different exposure times. Huang et al. (35) tested contact lens solutions as potential storage media for avulsed teeth. These authors recommend not using contact lens solutions for transporting exarticulated teeth in any emergency situation. Other solutions tested as potential transport media include milks of different fat contents (33), long shelf-life milk (36), baby formulas or evaporated milk (37), conditioned medium (38–40), Viaspan and Custodiol (organ transplant media) (30, 41), and egg albumen (42).

None of the above studies examined the ability of cells to recover from exposure to the various solutions. Examination of cell recovery after various treatments is important in understanding and optimizing the regenerative capacity of the PDL cells remaining on the avulsed tooth.

To test the ability of cells to survive and recover from exposure to potential storage solutions, we proposed: (i) to assess the viability of human PDL cells after exposure to different multipurpose single-bottle contact lens solutions, Gatorade®, HBSS, and milk at room temperature and on ice, and (ii) to examine the ability of the cells that initially survived the treatment to proliferate over extended periods of time (cell recovery assay).

Material and methods

Isolation of PDL cells from tooth segments

Human teeth extracted because of clinical necessity were obtained with the written consent of human subjects, according to the Baylor College of Dentistry IRB and NIH guidelines, and were immediately placed in individual 50 ml tubes containing 30 ml of sterile 1×PBS (VWR/EM Science, West Choster, PA) plus 5 ml of stock antibiotic (5200 units of penicillin and 5000 μl ml^{-1} of streptomycin, Invitrogen, Carlsbad, CA). Under a sterile hood, each tooth was removed from its tube and carefully placed in an adjustable clamp apparatus. The teeth were held by their crowns, with the roots facing upward. Sections of 0.5 mm thickness were removed from the apical and mid-root areas, using a diamond disc and an irrigating low-speed handpiece (1×PBS used as an irrigant) (Fig. 1A). Each tooth section was placed in a well of a 24-well plate, containing 2 ml of minimum essential culture medium (MEM; Life Technologies, Grand Island, NY, USA) plus 10% fetal bovine serum (FBS; Equitech-Bio Inc, Kerrville, TX, USA). All plates were placed in a humidified incubator at 37°C with 5% CO_2 overnight. The medium was replaced with fresh prewarmed MEM + 10% FBS after 24 h. Thereafter, the medium was changed every other day. Proliferative cells were grown and passaged when they filled greater than three-fourths of the well (Fig. 1B). For all the experiments, cells were plated at 5×10^4 cells per well and allowed to settle overnight. Each experiment had four wells per group, per time point. Each group had cells exposed for 1 h, with the plates being placed on the countertop at room temperature or on ice.

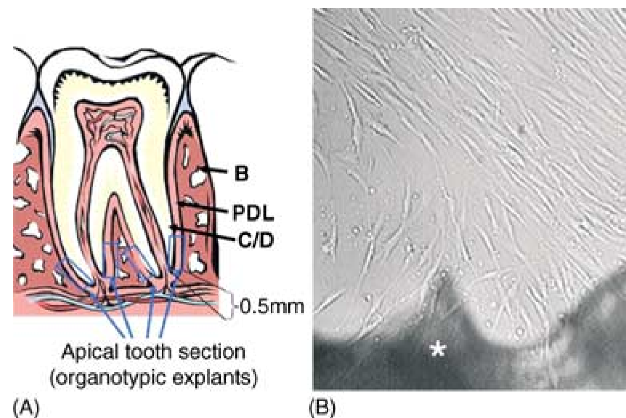

Fig. 1. Isolation and characterization of PDL. (A) Drawing of a cross-section through the jaw and a tooth. Tooth slices (rectangles) were made in the apical region of the extracted tooth to avoid any contamination with gingival epithelial cells. The tooth slices had dimensions of a depth of 0.5 mm, a width of 1–4 mm, and a height of 2–5 mm; variation was because of the topography of the tooth and differences in circumference. The tooth segments always consisted of cementum (C), PDL and dentin (D). (B) Proliferative PDL cells were then cultured *in vitro* from the tooth explants. Greater than 80% of the cells cultured had a spindle shape resembling the morphological properties noted for human PDL cells. The darker region at the bottom of the image (★) is the outline of a tooth slice.

Exposure of PDL cultures to different solutions

On the day of treatment, the culture medium was drained from each well, and the cells were exposed to 2 ml of the different experimental solutions, either at room temperature or on ice. The storage solutions used in the experiments were: (i) culture medium (90% Dulbecco's minimum essential medium (DMEM)) (Life Technologies, Grand Island, NY, USA), and 10% FBS; (ii) Milk (Borden® 2% reduced fat milk, fortified with vitamins D and E); (iii) HBSS; (iv) contact lens solution 1 (Sol 1) (Soft Wear; CIBA VISION Corp., Duluth, GA, USA); (v) contact lens solution 2 (Sol 2) (Opti Free; ALCON Laboratories Inc., Fort Worth, TX, USA); (vi) contact lens solution 3 (Sol 3) (Solo Care; CIBA VISION Corp., Duluth, GA, USA); (vii) tap water; and (viii) Gatorade®.

After exposure, the solutions were removed, the wells washed twice with 2 ml of sterile 1 × PBS, 2 ml of fresh, prewarmed growth medium was added, and the plates were returned to the incubator.

Assessing the viability of cells by trypan blue exclusion

Following exposure, the medium was removed from the wells after 0, 24 or 48 h, the cells were washed with 2 ml of sterile 1 × PBS, 100 μl of 0.25% trypsin was added, and the plates were incubated at 37°C for 5–10 min. Full growth medium (50 μl) and 0.4% trypan blue (50 μl) were added to each well, and the plates were returned to the incubator for another 5 min. After this time, a 20-μl aliquot was removed and placed under a coverslip on a hemocytometer, and both the viable and the non-viable cells were counted under the microscope. Each experiment was repeated at least thrice. The results were stored in an Excel file and statistically analyzed using the Graph Pad Prism software. Statistical significance was determined using either a one-way ANOVA and a post hoc Bonferroni test or a Student's *t*-test. Significance was indicated if $P < 0.05$.

Results

Room temperature

At the 0 h time point, the culture-medium group retained almost 67% of cells, significantly more ($P < 0.001$) than all the other solutions (Table 1; Fig. 2A). HBSS also contained a significantly ($P < 0.05$) larger number of vital PDL cells (37%) than the remaining solutions. Milk and the contact lens solutions were not statistically different from each other. Gatorade® had 8% of cells remaining, but was only statistically different from Sol 3 ($P < 0.01$). Water had the lowest numbers of vital cells, significantly lower than all the other solutions ($P < 0.001$),

Table 1. Percentage of the original number of cells at different time points at room temperature

	0 h (%)	24 h (%)	48 h (%)
Culture	66.8	45.4	77.8
Milk	19.8	5.8	18.2
HBSS	36.6	16.8	50.2
Sol 1	17.2	5.8	26.4
Sol 2	20.0	19.4	32.4
Sol 3	23.2	22.8	38.6
Water	1.8	0.4	0.6
Gatorade®	8.2	5.0	11.2

Percentages were calculated as follows: number of viable cells counted at the specified time points/original number of cells plated × 100.

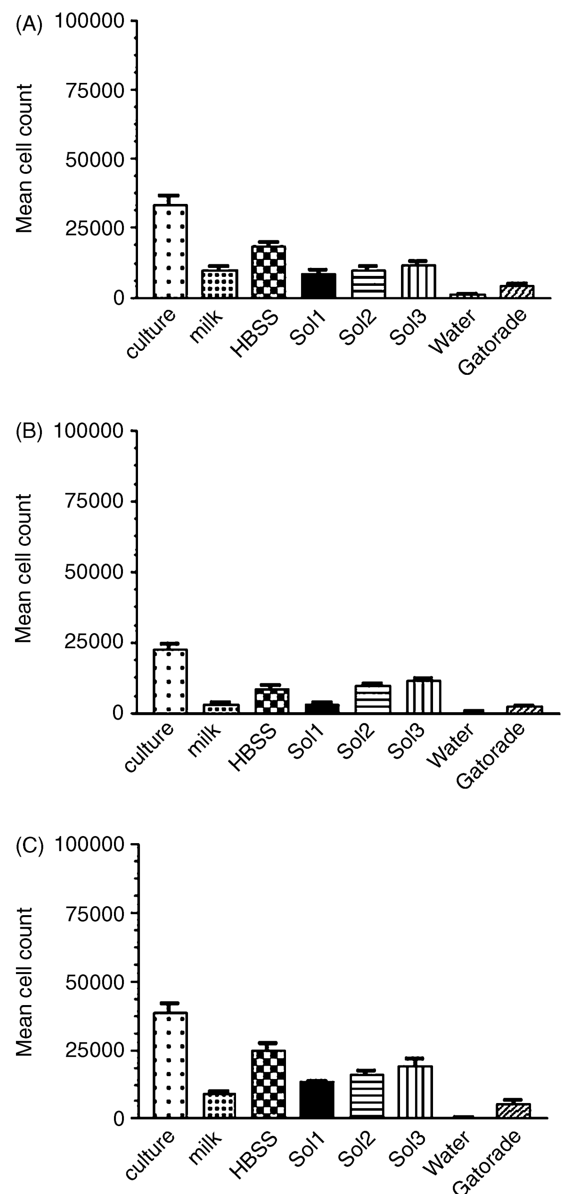

Fig. 2. Graphs showing the mean cell counts for PDL cells exposed to the experimental solutions at room temperature. (A) 0 h, (B) 24 h, and (C) 48 h after 60 min of exposure.

with the exception of Gatorade®. There was no significant difference between water and Gatorade® (Fig. 2A).

At 24 h, the mean number of live cells in all solutions was significantly lower than that at the 0-h time point, with the exception of contact lens solutions: Sol 2 and Sol 3 (Fig. 2A,B). The culture medium had significantly more viable cells than all the other solutions ($P < 0.001$) (Fig. 2B). HBSS, Sol 2, and Sol 3 had comparable numbers (17–23%) and were not significantly different from each other, but these solutions had significantly more viable cells than milk, Sol 1, Gatorade®, and water, which were not statistically different from each other. Water had almost no viable cells present (Table 1).

At 48 h, the mean cell count increased in all groups (Fig. 2A,C). The increase was statistically significant in all groups, except in water. The culture medium group had the highest number of cells (77.8%) ($P < 0.001$), followed by HBSS (50.2%), which had significantly higher numbers than all the other groups, except Sol 3 (Fig. 2C). The contact lens solutions, Sol 2 and Sol 3, had significantly more cells than the milk group, and all three had significantly more cells than Gatorade®. Gatorade® had the lowest numbers of viable cells except for water (Fig. 2C).

Ice

At 0 h, after exposure to the solutions, the culture medium group had the highest number of viable cells among the test groups (29.4%) (Table 2; Fig. 3A). These numbers were significantly higher than all three contact lens solutions, Gatorade®, and water, but not significantly different from HBSS and milk. The HBSS group, the milk group, the contact lens solutions groups, and the Gatorade® group did not have significantly different numbers of cells (Fig. 3A). The water group had the lowest number of PDL cells, with significantly fewer cells than the culture medium, HBSS, and milk groups (Fig. 3A).

Twenty-four hours after exposure to the experimental solutions on ice, the mean number of viable cells

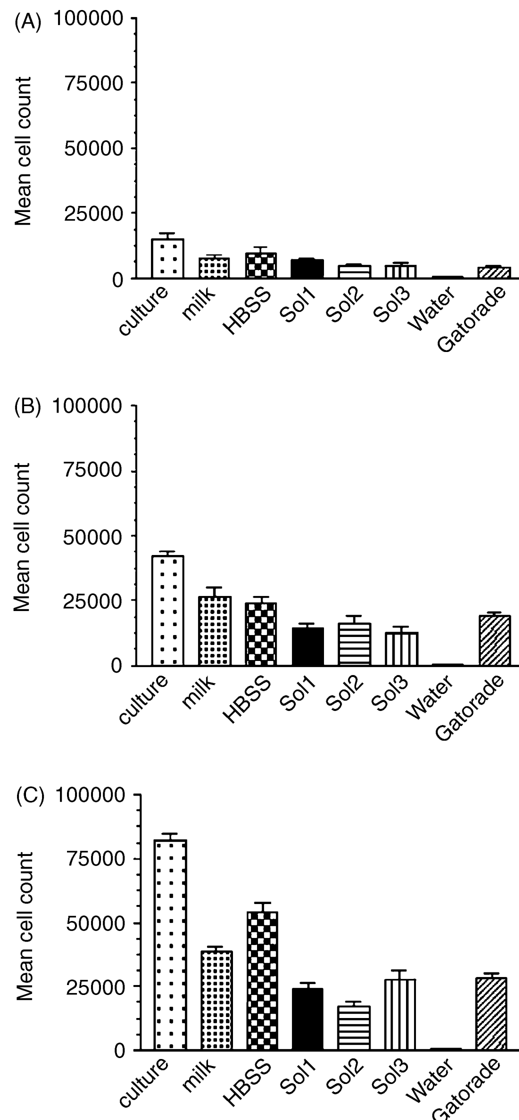

Fig. 3. Graphs showing the mean cell counts for PDL cells exposed to the experimental solutions on ice. (A) 0 h, (B) 24 h, and (C) 48 h after 60 min of exposure.

in all the groups was higher than that at 0 h (Fig. 3A,B). With the exception of Sol 3 and water, this increase was statistically significant in all groups ($P < 0.05$). At 24 h, the culture-medium group was found to have significantly higher number of cells than the other groups ($P < 0.001$), whereas the water group had the lowest number of cells (Fig. 3B). The mean number of viable PDL cells in the milk group was slightly higher than that in the HBSS group ($P > 0.05$), but both these groups had significantly higher number of cells than the contact lens solution groups (Table 2). All three contact lens solution groups had numbers of cells comparable to one another and to Gatorade®.

Forty-eight hours after exposure to the experimental solutions, higher mean cell counts were noted in all groups compared to 24 h (Fig. 3B,C). With the

Table 2. Percentage of the original number of cells at different time points on ice

	0 h (%)	24 h (%)	48 h (%)
Culture	29.4	83.8	164.6
Milk	17.0	53.6	77.4
HBSS	19.6	48.6	107.6
Sol 1	13.8	29.2	48.4
Sol 2	9.0	32.6	34.0
Sol 3	9.2	25.8	55.6
Water	1.0	0.8	0.8
Gatorade®	8.4	38.6	56.8

Percentages were calculated as follows: number of viable cells counted at the specified time points/original number of cells plated × 100.

exception of Sol 2 and water, this increase was statistically significant. The culture medium and HBSS groups had significantly higher numbers of cells ($P < 0.001$) than all the other groups (Fig. 3C). The milk group had significantly higher numbers of viable cells than the Sol 1 and Sol 2 groups ($P < 0.01$) and the water group ($P < 0.001$), but not higher than the Sol 3 ($P > 0.05$) and Gatorade® ($P > 0.05$) groups. The contact lens solution and Gatorade® groups had similar numbers, although Gatorade® had significantly more cells than Sol 2 ($P < 0.05$). The water group had significantly fewer viable cells than all other treatment groups ($P < 0.001$).

Discussion

In the present study, we tested the ability of various aqueous solutions to maintain the viability and proliferative potential of human PDL cells at room temperature and on ice. The ability to proliferate is critical to the regeneration of oral tissues after damage. Significantly higher number of cells survived and proliferated when the exposure was performed on ice compared to room temperature. Water had a detrimental effect on PDL viability at either temperature, whereas HBSS preserved significantly more viable PDL cells than any other experimental solution (with the exception of culture medium). Contact lens solutions and Gatorade® consistently preserved more viable cells than tap water at both temperatures, whereas in the milk groups, only ice-cold milk was good as a preservative of the PDL cells' proliferative ability.

Effects of temperature on viability of stored PDL cells

Several studies have shown that the temperature of the storage medium affects the viability of the PDL cells (19, 22, 47, 58). The number of viable cells in all the groups exposed to ice was less at the initial time point than in the groups exposed to room temperature. Culture after exposure showed that the cells quickly recovered, multiplying to or exceeding the original numbers plated in some groups. The low numbers of cells we observed immediately after exposure to ice is likely caused by the shock of the sudden change from body temperature to ice-cold temperature. Cold sometimes causes cells to detach from the culture plates, reducing the number of viable cells counted. In contrast, when the experiment was performed at room temperature, the initial cell counts were higher but a considerable, and in most cases, significant drop in the number of viable cells was consistently observed after 24 h. This was followed by a subsequent increase in cell numbers to levels slightly higher than those observed after the initial exposure but still lower than those on ice. One explanation could be that some of the cells that were viable immediately after exposure were in the process of dying, resulting in fewer viable cells, during subsequent culture. From these data, it can be concluded that storage on ice provided conditions that enabled a higher number of cells to remain viable and healthy and thus functional. Based on these observations, we believe that storage of exarticulated teeth in a physiologic medium on ice for up to 1 h provides better conditions than storage for the same time and in the same medium at room temperature.

Viability of PDL cells in various solutions

All the solutions tested exhibited significantly better cell viability than storage in regular tap water. Both at room temperature and on ice very few cells exposed to water survived, and cells that survived were not able to recover after additional culture, consistent with previous reports (19, 25, 51).

Hanks balanced salt solution is a standard saline solution, which is widely used in biomedical research to support the growth of many cell types (52). It is non-toxic, pH-balanced, and contains many essential nutrients (30, 34). HBSS has an osmolality that ranges from 270 to 320 mOsm (52). Cell growth occurs in a range of 230–400 mOsm; however, growth is optimal in a range of 290–330 mOsm (26, 53). The results of the present study demonstrated that significantly more viable cells survived in HBSS in comparison to any other experimental solution, with the exception of culture medium, both at room temperature and on ice. The cells stored in HBSS were able to survive and proliferate better than those in the other treatment groups. These results are in agreement with several previous investigations (19, 29, 30, 54). A disadvantage of HBSS is that it may not be readily available in many locations in which tooth avulsions are likely to occur. Recently, a tooth preserving system utilizing HBSS as a storage medium was developed and has become commercially available as Save-A-Tooth™ (Save-A-Tooth Inc., Pottstown, PA) (52), although it is not yet widely available in pharmacies or drug stores.

The improved viability of cells stored in milk may be caused by the physiologic osmolality of milk (19, 55), the cytoprotective effects of other nutrient constituents, as well as the pH buffering system in which cells can survive for long periods of time (56). Two per cent reduced fat milk was used instead of whole milk, because there is evidence that milk with a lower fat content may be more appropriate at maintaining cell viability than milk with a higher fat content (33). Our results agreed with those of the previous studies that suggested milk could be used as a storage medium for PDL cells, but only when stored on ice.

Hanks Balanced Salt Solution and milk may not be readily available in locations where avulsions usually

occur, especially within an athletic environment (e.g. games, gyms, athletic fields, etc.). A potential transport medium that is commonly found at sporting events is the oral rehydration fluid Gatorade®. According to the Exercise Physiology Laboratory at the Gatorade Sports Science Institute, Gatorade® Thirst Quencher has a pH of 3 and osmolality ranging from 280 to 360 mOsm l^{-1}, depending on the shelf life of the product (33). Gatorade® preserved more viable cells than tap water, both at room temperature and on ice. Previous studies suggested that the viability of the PDL cells stored in Gatorade® was comparable to dry storage or storage in tap water (33, 34), but it must be noted that those studies were performed at or near 37°C, which results in elevated cellular metabolism and production of by-products that could be toxic to the cells. However, Gatorade® preserved significantly more cells when stored on ice than at room temperature. Therefore, Gatorade® on ice could serve as a short-term storage medium for avulsed teeth in cases where more acceptable media like HBSS or milk are not readily available.

According to the American Optometric Association (2002), nearly 25 million Americans wear contact lenses; 50% are of the age between 25 and 44 years, and 10% are under the age of 16 years. The number and the age of the individuals who wear contact lenses make using contact lens solutions for preserving the teeth after avulsion injuries practical because these solutions are available at school premises or athletic facilities, where most such injuries occur. The most popular types of solutions today are the multiple purpose solutions. These solutions have been approved as single-bottle systems for cleaning and disinfecting contact lenses (57). Three different single-bottle systems, Soft Wear and Solo Care (CIBA VISION) and Opti Free (ALCON), were examined here as potential temporary storage media for avulsed teeth. They contain buffered, isotonic saline solutions with the addition of preservatives. According to the manufacturer, these solutions are specifically designed to store the lenses, which are then placed onto the eye without the need of prior rinse with saline or water, implying that they are not detrimental to the sensitive ocular tissues. No significant differences in the ability of the different contact lens solutions to maintain cell viability were found. They preserved significantly more viable cells than tap water both on ice and at room temperature, and significantly more cells than Gatorade® at room temperature. Storage of the PDL cells in Opti Free and Solo Care also proved to be better than storage in milk at room temperature. However, storing the cells in ice-cold milk was more beneficial than any of the contact lens solutions. Based on these results and within the limitations of this study, the contact lens solutions examined here could be utilized as temporary storage media for avulsed teeth, when more acceptable storage solutions like HBSS or ice-cold milk are not available. These conclusions disagree with those of Huang et al. (35). Their recommendation was to avoid contact lens solutions as a substitute for saline in any emergency situation. However, they counted the number of viable cells immediately after exposure to the different solutions by direct observation and from photographs. In the present study, cell recovery was assessed after exposure, and it demonstrated that the cells that survived the exposure were able to multiply.

The PDL cells used in this study were obtained from human teeth extracted for therapeutic reasons. A small number of polygonal epithelial cells were found in the cultured samples. Blomlof & Otteskog (22) also observed epithelial cells in explant cultures of human PDL cells, although migrating fibroblasts tended to predominate and overgrow the epithelial cells. Blomlof (19) and Huang et al. (35) verified these observations and included both cell types in their cultures, as was performed in the current study.

The fibroblast-like cells, which are mainly responsible for the production and maintenance of the connective tissue in the PDL, have been shown to have collagen synthesis rates about fourfold higher than that of human skin fibroblasts (43). They are able to synthesize types I, III, and IV collagen (44), and their unusually high turnover rate of collagen has been suggested to be a result of mechanical stress caused by occlusal forces (45). Previous investigators (30, 42) have used fibroblasts isolated from sources other than the PDL. Despite their similar morphology, lip, gingival, or skin fibroblasts do not behave in a similar manner in culture as the PDL fibroblasts. Protein and collagen production rates are significantly greater in PDL cells (43), alkaline phosphatase levels are higher (45), and growth rates are also significantly different (46). Therefore, fibroblast cells obtained from a source other than the PDL may not accurately reflect the ability of PDL fibroblasts to remain viable in culture (36).

We chose the trypan blue exclusion staining technique because it is quick, easily performed, and distinctively differentiates non-viable cells from viable cells. However, the health of the viable cells and their ability to proliferate cannot be determined from this technique. Thus, we determined cell recovery by counting the increase in the number of cells after exposure to the different solutions. This extra step differentiates our study from similar studies in the past, where the number of viable cells was determined only after exposure to different experimental solutions. This renders our *in vitro* study clinically more relevant because it differentiates between cells that are viable immediately after exposure but damaged to the point of being mitotically inactive or apoptotic and so

unable to repopulate the damaged tissues. Consistent with this idea, recent studies showed that *in vitro* proliferation assays are much more sensitive for measuring cell viability because of extra-oral treatment times and storage conditions rather than dye exclusion or cell attachment techniques (47).

Experimental exposures were performed immediately after cell attachment was verified to obtain an accurate count of the number of cells plated. Thus, the number of viable cells counted at each time point could be calculated as a percentage of the number of cells originally placed in each well. PDL cell counts immediately after treatment were lower than those previously reported (19, 22, 25, 26, 48–50). In previous experiments, the cells were plated and then cultured to confluence over a number of days prior to the initiation of the experiment, thus increasing the initial number of treated cells because of cell proliferation during this time period. Therefore, the number of viable cells counted after the exposure to the experimental solutions did not represent a percentage of the original number of cells plated but instead, a percentage of the increased number of cells, which was not determined in any of these studies.

The viability of PDL cells after exposure to various solutions for 1 h was used as a measure of these solutions' effectiveness as potential transport media. Ideally, an *in vivo* study should be designed to test the same solutions in a manner that would be clinically more relevant. Teeth, intentionally extracted and stored in these solutions for different amounts of time could be replanted, and the tissue reactions could be observed histologically to determine the rate of periodontal healing, resorption, and ankylosis. Alternatively, if the same *in vitro* study was to be performed in the future, several exposure times could be added in order to examine the effects of both short- and long-term storage in the experimental solutions. In addition to the trypan blue exclusion technique and the cell recovery assay, a cell apoptosis assay could be used to more accurately determine the condition of the cells after their exposure to the different solutions.

In conclusion, our study supports the evidence that storage of an avulsed tooth on ice is more beneficial than storage at room temperature. HBSS is the optimal solution for storing avulsed teeth, consistent with previous results. The use of contact lens solutions and Gatorade® at room temperature and water in any situation should be avoided. If ice is available, low-fat milk is an appropriate alternative to HBSS for storage of avulsed teeth. Either single bottle contact lens solutions or Gatorade® on ice could serve as short-term storage media (1 h), if the other solutions are not readily available.

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