Endod Dent Traumatol 1999; 15: 149–156
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Endodontics & Dental Traumatology
ISSN 0109-2502

In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in six different media

Ashkenazi M, Sarnat H, Keila S. *In vitro* viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in six different media. Endod Dent Traumatol 1999; 15: 149–156.
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Abstract – The choice of storage medium for preserving traumatically avulsed teeth is important for the success of future replantation. The objectives of this study were to evaluate the effectiveness of six different media: culture medium, α minimal essential medium (α-MEM), milk, Hank's balanced salt solution (HBSS), ViaSpan and conditioned medium (CM) to preserve cultured periodontal ligament fibroblasts (PDLF). Periodontal ligament fibroblasts were obtained from explants of human healthy extracted teeth. Plates with confluent PDLF were soaked in the various media for 2, 8 and 24 h at 4°C. A control group was incubated with culture medium at 37°C. After incubation, cell viability was determined by trypan blue exclusion test. Viable cells were then analyzed for mitogenic (with thymidine) and clonogenic capacity (by culturing one cell/well). Storage of PDLF up to 24 h decreased their vitality by only 2%–14%. Vitality of the PDLF after 2, 8 and 24 h was highest when stored in milk or HBSS (91%–97%) and lowest when stored in ViaSpan or CM (82%–93%). PDLF stored for 2–8 h in various media had a mitogenic capacity comparable to the control. However, increasing the storage period to 24 h decreased the mitogenicity of the cells by 3%–39%. The highest mitogenicity was found in PDLF stored in milk or HBSS and the lowest in CM or ViaSpan. The clonogenic capacity of the cells dropped by 38%–71% after 24 h and was the best indicator of the deteriorating effect of long storage. Milk and HBSS were the most effective in preserving the clonogenic capacity. Nevertheless, reduction in the viability, mitogenicity or clonogenic capacity was statistically significant in nearly all the tested media only after 24 h of incubation. In conclusion, HBSS and milk were the most effective media for preserving the viability, mitogenicity and clonogenic capacity after storage for up to 24 h at 4°C.

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Key words: avulsion; clonogenic capacity; mitogenic capacity; PDL fibroblasts; storage medium

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Accepted February 16, 1999

Traumatic injuries to the permanent anterior teeth occur mainly in the 7- to 10-year age group, of which 0.5%–16% result in tooth avulsion (1). Successful replantation of the avulsed tooth depends on the existence of viable periodontal ligament fibroblasts (PDLF) that are capable of proliferating over the denuded damaged root areas (1–3). This can be achieved by replantation within 30 min after avulsion or by placing the tooth in storage medium until the patient can be seen by a dentist for replantation (4–6). The optimal storage medium should be able to preserve the viability, mitogenicity and clonogenic capacity of the

injured PDLF and their progenitors. This is essential in order to quickly repopulate the denuded root surface by PDLF and prevent osteoclasts from attaching to this area.

In the past, several media have been evaluated for storage of avulsed teeth. Tap water has been shown to be almost as harmful to the PDLF as dry storage (3). Saliva and saline have been recommended for many years as storage media for exarticulated teeth, but only for a short time (7–9). Storage of cultivated PDLF for 2–3 h in saliva results in almost total cell death, since saliva is very hypotonic and contains numerous bacteria (4, 10). At present, the storage medium of choice for avulsed teeth is milk, which has a relatively physiological osmolality with lower bacterial content than saliva (6, 8, 9, 11–13). Milk has been shown to be effective as a storage medium for up to 12 h (11). Its main advantage is the common availability.

In the 1970s several studies have shown that culture medium (Eagle's medium at 37°C) can preserve PDLF for extended periods before replantation (3, 14–16). Viable cells can be trypsinized and cultured from teeth preserved in culture medium (37°C) as long as 1 year after extraction (14).

Another popular and very promising storage medium is Hank's balanced salt solution (HBSS), which has a pH of approximately 7 and an osmolarity of 270 to 290 mOSM/L. This medium has been shown to be effective for preserving avulsed teeth for extended periods of time (17–19).

One reason that these media have not been recommended to the public is that they are seldom available near the site of an accident. Therefore, the concept has been thought to be of academic interest only. Recently, a tooth preserving system that uses HBSS as a storage medium has become commercially available for storing avulsed teeth until replantation. The aim is to make these preserving systems available at schools, sports facilities, ambulances, hospital emergency rooms, or any place where avulsion might occur, including the home. With this tooth preserving system, the use of a variety of storage media becomes practical, with the possibility of maintaining the vitality of the periodontal ligament cells (PDL) of the avulsed tooth for an extended period (20).

ViaSpan, one of the suggested storage media, is an extremely effective cold storage medium for organs before transplantation (21). Its osmolarity is 320 mOSM/L, with a pH of approximately 7.4, which is ideal for cell growth. The viability of human lip fibroblasts following storage in milk, HBSS and ViaSpan at room temperature has been evaluated (17). All of these media gave comparable results up to 6 h; however, after 12 h, the effectiveness of milk dropped dramatically, being ineffective after 48 h of storage. HBSS and ViaSpan were extremely effective for the first 24 h. After 7 days, ViaSpan was the most effective storage medium.

Another recommended storage medium is conditioned medium (CM) (19). It is the supernate of non-confluent PDLF harvested after 2 days of culturing. The idea was that this storage medium will not only preserve the viability of PDLF of avulsed teeth, but will also stimulate their growth due to the presence of growth factors released to the supernate during the exponential growth of the fibroblasts.

The effectiveness of the storage media depends on the type of medium and the length of the extra-alveolar time. The longer the storage period, the more important it is to use the optimal medium. Various *in vitro* assays have suggested evaluating the effectiveness of the storage media. Conceivably, these assays may provide useful markers for cell survival *in vivo* after tooth replantation. However, previous reports indicate a weak relationship between various *in vitro* measures of cell viability and clinical success rates (19, 22). Recently, it has been shown that *in vitro* assays of clonogenic capacity are much more sensitive to extra-oral storage time and storage conditions than dye inclusion or cell attachment (12).

The purpose of this *in vitro* study was to compare the viability, mitogenicity and clonogenic capacity of PDLF stored in six different storage media.

Material and methods

General procedure

Cultured human PDL were placed in 30 mm petri dishes and grown to confluent at 37°C in culture medium composed of α-MEM supplemented with 15% fetal calf serum (FCS) and antibiotic solution (AB) consisting of penicillin (100 units/mL), gentamicin (50 μg/mL) and fungizone (0.3 μg/mL). At the start of the experiment, the culture medium was suctioned, and 4 mL of the tested storage medium was added to each plate. The plates (two dishes in each group) were kept at 4°C for 2, 8 and 24 h. Control dishes were incubated at 37°C in humidified air containing 7% CO_2 with culture medium. After 2, 8 and 24 h, the medium was suctioned, the cells were washed (three times in phosphate-buffered saline containing Ca^{++} Mg^{++} [PBS]) and trypsinized (1 mL 0.025% trypsin+0.02% EDTA). The detached cells were harvested to a test tube, centrifuged (250×g, 5 min, 15°C) and the pellet was re-suspended in 2 mL of fresh culture medium. The cells were counted in a hemocytometer and the viability was determined by trypan blue exclusion test (23). Cells from each plate were counted three times and the average of the counts recorded. The viable cells were then analyzed for mitogenic and clonogenic capacity as described below.

Periodontal ligament cells

Cultures of human PDL cells were initiated by explantation and maintained in our laboratory as previously described (24). Periodontal ligament fibroblasts were obtained from explants of a fully erupted premolar or molar tooth extracted from young adults for non-periodontal reasons. Following extraction, the tooth was transferred to a test tube containing 10× the previous antibiotic concentration. Under sterile conditions, the PDL tissue was mechanically removed by scraping the middle third of the root surface with a sharp blade. These tissue explants were placed in 25 cm^2 tissue culture flasks containing culture medium. The cells were incubated at 37°C in humidified air with 7% CO_2 for 2–4 weeks. The medium was replaced every 2–3 days until sufficient cell proliferation was obtained. Trypsin/EDTA incubation was used to harvest the cells, which were transferred into 75 cm^2 flasks for continued growth. Cells were used after three to five passages.

Storage media

The media examined were low-fat milk, culture medium, α-MEM (Biological Industries, Beit Haemek, Israel), HBSS (Biological Industries, Beit Haemek), ViaSpan (No. 1000–16–06, Du Pont, Wilmington, DE, USA), and conditioned medium (CM). The low-fat pasteurized and homogenized milk had a content of 3 g protein, 1 g lipid, 5 g carbohydrate, 120 mg calcium and 50 mg sodium per 100 g. The culture medium was composed of α-MEM supplemented with 15% FCS (Biological Industries, Beit Haemek) and antibiotic solution (AB) consisting of penicillin (100 units/mL), gentamicin (50 μ/mL) and fungizone (0.3 μg/mL) (Biological Industries, Beit Haemek). Conditioned medium was achieved by culturing PDLF in a concentration of a half confluent flask for 2 days. The supernate was then harvested and used or stored at −16°C until use.

Hank's balanced salt solution was composed of 8 g/L sodium chloride, 0.4 g/L D-glucose, 0.4 g/L potassium chloride, 0.35g/L sodium bicarbonate, 0.09 g/L sodium phosphate, 0.14 g/L potassium phosphate, 0.14 g/L calcium chloride, 0.1 g/L magnesium chloride and 0.1 g/L magnesium sulfate (Biological Industries, Beit Haemek).

Mitogenic assay

The mitogenic capacity of cells represents the functional ability of the entire cell population (not just the progenitor cells) to attach to a solid surface and to proliferate. After incubation with the examined medium, cells from each plate were washed (three times in PBS), trypsinized, centrifuged (5 min at 250 g) and re-cultured in six wells of 24-well-plates in concentrations of 5×10^4 viable cells/well. After 24 h, the medium with the unattached cells was removed and fresh culture medium supplemented with 2 μCi/mL H^3 thymidine (5 Ci/mMol, Rotem Industries, Beer Sheva, Israel) was added to each well. Following 6 h incubation (37°C, 7% CO_2), the cells were rinsed (three times in PBS) and solubilized in 200 μL of 0.1% sodium dodycyl sulfate (SDS, No-L-4509, Sigma, St. Louis, MO, USA). The radioactivity was determined in a counter (Packard Tricarb 4530, United Technologies, Packard, IL, USA). This assay was repeated six times. In five experiments, two plates (12 wells) of cultured cells were used for each tested medium.

Clonogenic capacity

The clonogenic capacity of PDLF is an estimate of the proportion of progenitor cells in a population with extensive proliferative and colony-forming capacities (12–13). This may suggest the effectiveness of each media to preserve the progenitor cells. To evaluate the clonogenic capacity of the stored cells, cells were inoculated into 96-well-plates at a concentration of one viable cell/well. For each tested group, 96 replicates were plated and grown for 3 weeks in a culture medium at 37°C in humidified air containing 7% CO_2. Under these conditions, discrete colonies arising from a single cell that covered 70% to 100% of the well were counted under light microscopy (×200). The percentage of cells with clonogenic capacity was calculated as the number of colonies formed/number of cells seeded ×100. Thus, the clonogenic capacity reflected the likelihood that each colony arose from a single cell. The experiments for 2 and 8 h were repeated three times (three 96-well-plates), and for 24 h, five times (five 96-well-plates).

Statistical analysis

Differences among the various media (milk, HBSS, culture medium, α-MEM, CM and ViaSpan) and among various incubation periods (2, 8 and 24 h) were examined. The differences in the proportions, viability and clonogenic capacity were evaluated by the chi-square test (γ^2) and the differences in the radioactive thymidine incorporation (mitogenic capacity) were evaluated using the Wilcoxon rank sum test.

Results

The results of the vitality tests are summarized in Table 1. Storage for 2 h significantly decreased the vitality when compared to the control group only in cells stored in CM or ViaSpan (3%; $P=0.0015$ and

Table 1. Viability of PDLF after incubation in various storage media for different time periods

	Incubation time		
	2 h	8 h	24 h
Control	95.4±1.4	95.4±1.4	95.4±1.4
Milk	96.8±1.2	94.7±2.0	93.1[a2]±2.5
HBSS	95.0±1.5	95.0±1.0	90.8[a2,8]±3.1
Culture medium	94.4±1.8	95.6±0.9	86.1[a2,8]±3.4
CM	92.3±1.8	88.7[b,a2]±3.0	86.5[a2]±2.5
ViaSpan	92.8[b]±1.9	86.7[b,a2]±3.7	82.0[a2,8]±4.3
α-MEM	95.9±0.5	92.1[b,a2]±2.4	87.3[a2,8]±2.9

Results represent the mean±SEM of six experiments (11 different plates).
[a] Significant differences between different incubation times (2 h, 8 h).
[b] Significant differences when compared to the control.

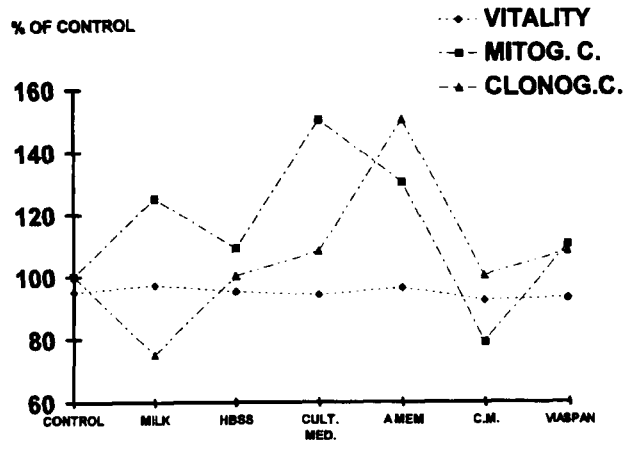


Fig. 1. Functional abilities of PDLF after 2 h of storage.

2%; $P=0.0064$, respectively). Storage for 8 h significantly reduced the viability when compared to the control group in cells stored in CM, ViaSpan, or α-MEM (4%–7% reduction, $P=0.00005$, $P=0.0064$ and $P=0.0007$, respectively). Increasing the storage period to 24 h led to statistically significant reductions in all tested media (2%–14%, $P<0.0003$).

The most profound decrease was observed in the ViaSpan group, where the reduction was about 12% ($P<0.00001$). The best media for preserving the relative viability of the cells for up to 24 h at 4°C were milk and HBSS, which showed a reduction of only 2% and 5%, respectively. Milk and HBSS preserved the viability of stored PDLF significantly better than CM or ViaSpan after 2, 8 and 24 h of storage ($P<0.00001$ after 8 and 24 h, and non-significant, $P<0.4$, after 2 h). Microscopically, cells stored in α-MEM and in culture medium showed higher aggregation after 24 h of soaking.

The results of the mitogenicity tests are summarized in Table 2. The ability of the stored cells to proliferate was compared to the mitogenicity of cells incubated in culture medium at 37°C, 5% CO_2. After 2 h of storage, the cell mitogenicity was higher by 10%–50% compared to the control group in all tested media, except CM (−21%, $P=0.1$) (Fig. 1, Table 2). After 8 h of storage, the mitogenic capacity of the PDLC dropped by 2%–10% compared to the control and by 8%–35% compared to the mitogenic capacity of the cells after 2 h of storage. Cells stored in ViaSpan had the lowest proliferative ability when compared to other tested media (90%, $P=0.19$). In contrast, cells incubated in milk and HBSS showed better proliferative ability than the control. However, after 2 and 8 h of incubation, these differences were not statistically significant ($P<0.2$–0.5). The mitogenic capacity of the cells dropped significantly after 24 h incubation by 16%–39% when compared to the control group (culture medium $P=0.03$, ViaSpan $P=0.14$, α-MEM $P=0.02$, CM $P<0.04$) and by 3%–44% when compared to their mitogenic capacity after 2 h of storage (α-MEM $P<0.00001$, culture medium $P<0.005$, CM $P<0.00003$, ViaSpan $P<0.00007$). The only groups that showed comparable results to the control

Table 2. Mitogenic ability of PDLF following incubation in different media compared to control cells

	Incubation time		
	2 h	8 h	24 h
Control	100	100	100
Milk	125.0±29.0	114.8±18.8	97.3±3.3
HBSS	109.1±18.1	106.7±10.2	101.7[a8]±16.8
Culture medium	149.7±26.7	98.0±9.6	83.6[b]±11.5
α-MEM	129.6±33.3	95.0[a2]±7.9	80.8[a8,b]±15.4
CM	78.5±16.2	99.0±21.0	76.0[b]±12.9
ViaSpan	109.5±32.0	90.0±18.3	61.3[a8]±28.6

Results represent the mean±SEM of four to six different experiments. In each experiment, 6–12 wells were evaluated.
[a] Significant differences between different incubation times (2 h, 8 h).
[b] Significant differences when compared with the control.

Table 3. Percentage of cells with high clonization capacity (3/4 to confluent) after 2, 8 and 24 h of incubation in different media

	Incubation time		
	2 h	8 h	24 h
Control	12.0±2.1	12.0±2.1	12.0±2.1
Milk	8.7±1.6	8.0±2.4	7.5±2.3
HBSS	12.4±1.5	11.4±1.6	7.4[a2]±1.7
Culture medium	12.7±1.8	10.7±1.7	5.5[a2,8]±2.1
α-MEM	17.9±2.7	12.0[a2]±2.2	4.2[b,a2,8]±2.1
CM	12.1±3.1	8.3±1.7	3.5[b,a2,8]±1.7
ViaSpan	12.9±2.8	13.9±3.6	4.2[b,a2,8]±1.7

The results represent the mean±SEM of three to six experiments (288–576 cells for each group).
[a] Significant differences between different incubation times (2 h, 8 h).
[b] Significant differences when compared with the control.

after 24 h of storage were HBSS and milk (102% and 97%, respectively). The lowest proliferative ability after 24 h was found in cells stored in CM or ViaSpan: 76% and 61%, respectively, (P=0.06).

The clonogenic capacity of PDLF after storage in six media for 2, 8, and 24 h is presented in Table 3. In general, the percentage of cells with clonogenic capacity was reduced with increased storage time of 8 to 24 h (Fig. 1–3). However, these reductions were statistically significant only after 24 h of storage (except in the milk group). The increased reduction in the clonogenic capacity of stored PDLF with increased storage duration was mostly expressed in this functional ability when compared to mitogenic or vitality tests (Fig. 2 and 3).

After 2 h of incubation, cells stored in all tested media had comparable clonogenic capacity to the control (P<0.3). After 8 h, this capacity dropped by 8–31%. The highest decrease, from 2 to 8 h, was noted in cells stored in α-MEM and CM (33% P<0.015, 31% P<0.17, respectively). All tested groups had comparable ability to preserve the clonogenic capacity after 8 h, except cells stored in ViaSpan where there was a statistically significant higher clonogenic capacity when compared with cells stored in milk (P=0.035) (Table 3). After 24 h of incubation, the clonogenic capacity of the cells was significantly lower by 38%–71% when compared to the control group (culture medium P<0.0007, ViaSpan P<0.004, CM P<0.0002, α-MEM P<0.0001, milk P<0.9, HBSS P<0.04) (Fig. 3). Cells stored in milk and HBSS had the highest clonogenic capacity when compared to other tested media (P=0.22 and P= 0.17, respectively), but had 38% lower clonogenicity then the control group (P=0.3). Statistically significant differences were found after 24 h of storage between HBSS and ViaSpan (P=0.025), HBSS and CM (P=0.0056), milk and CM (P=0.02), and between the control group and α-MEM, (P=0.0002), culture medium (P=0.053), CM (P<0.0003) and ViaSpan (P<0.0003) groups. Interestingly, the clonogenic capacity of PDLF stored in milk after 2 and 8 h was relatively low compared to other tested media. However, after 24 h, this capacity dropped slightly (14%, P<0.8), while in other media it dropped by 40%–77%. This means that cells stored in milk for 24 h had the highest clonogenic capacity (7.5%).

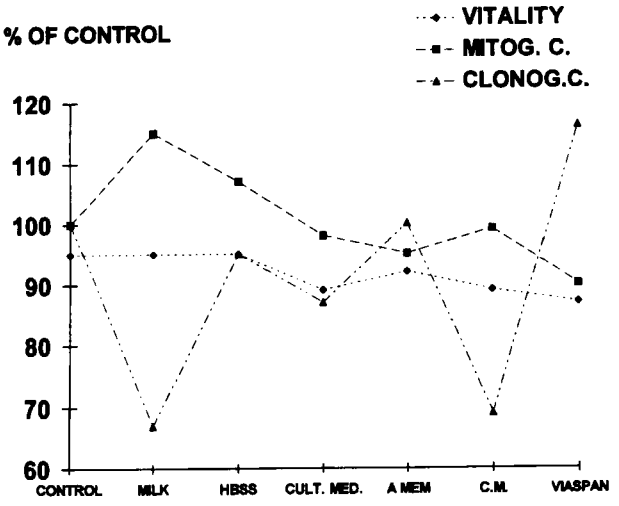


Fig. 2. Functional abilities of PDLF after 8 h of storage.

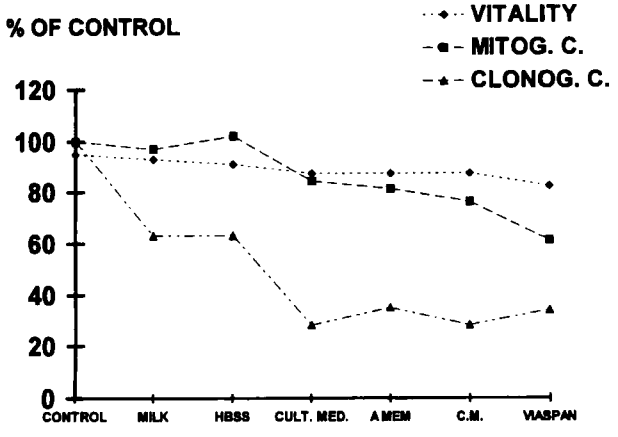


Fig. 3. Functional abilities of PDLF after 24 h of storage.

Discussion

Experimental design

This study was conducted in order to compare the effect of six accepted storage media on the functional abilities of periodontal ligament fibroblasts. Periodontal ligament fibroblasts from teeth of young adults were used to imitate the cell lining of avulsed teeth as closely as possible. The effectiveness of storage media was evaluated after 2, 8 and 24 h. These intervals were found to be clinically relevant. The PDLF were stored at 4°C and not at room temperature. This low temperature had the advantage of limiting bacterial growth and preventing milk from souring, which may be significant after reimplantation. Previous studies have shown that cooler temperatures reduce cell swelling, increase cell viability and improve recovery, factors that could promote healing (6, 10).

Functional abilities

The effectiveness of the tested media was evaluated not just by vitality, but also by the ability of the cells to function as manifested by the mitogenicity and clonogenic capacities of the PDLF after storage. In this study, there was no parallelism between these functional abilities. Thus, a medium that induced a relatively low viability and mitogenic capacity could induce high clonogenic capacity. Nevertheless, the

optimal medium should preserve most of the functional capabilities. The importance of one function over another in predicting clinical success should be further evaluated in clinical studies.

Viability

The viability of PDLF is essential for the clinical healing of replanted exarticulated teeth. In this study, the trypan blue exclusion staining technique was used. This is quick, easily performed and differentiates clearly between dead and viable cells. Cell recovery after storage in almost all of the experimental conditions was high (82%–97% viability). These results may be attributed to the origin of the cells, i.e., explants from very young adults, early passage (3–6, 25), low storage temperature (4°C), or the high quality of the currently tested media. However, this absolute high recovery showed a weak relationship to the low clinical success rate, which is in agreement with others (5, 22).

Mitogenic capacity

In this study, PDLF stored for 2–8 h in various media gave similar statistical results to the control. Moreover, storage of cells for 2 h (4°C) resulted in higher mitogenicity in all tested media, although these differences were not statistically significant. These results were in contrast to previous studies in which the proliferative capacity of PDLF decreased with longer storage times (19, 26). The relatively high mitogenicity of cells after 2–8 h could be explained by the priming and supportive effects of the low temperature (4°C) and by using only recommended storage media. The deterioration effect of prolonged storage periods on mitogenicity was statistically significant in this study only after 24 h.

Clonogenic capacity

Our study indicated that the clonogenic capacity of the cells was the best functional test to demonstrate the deterioration effect of prolonged storage times and the differences between groups. Nevertheless, the reduction in this ability was statistically significant in almost all test media only after 24 h of incubation. The large discrepancies between the clinical success of replanted teeth and the *in vitro* assays (after 2 and 8 h of storage), emphasized the importance of developing more sensitive *in vitro* assays to evaluate the differences between various storage media even after short periods of storage.

Storage media

Hank's balanced salt solution is a popular storage medium that has been shown to be effective in preserving avulsed teeth for extended periods (17–19). In this study, HBSS was the most effective storage medium in preserving the viability, mitogenic and clonogenic capacity of cells after storage of up to 24 h at 4°C.

Milk was the second best choice according to this study. This medium was extremely effective in preserving the vitality and mitogenic ability of PDLF for up to 24 h and matched the HBSS and control results, which were in agreement with other clinical and laboratory reports. Milk, as a storage medium, induced successful healing *in vivo*, even after 12 h (6, 11, 13) and better maintenance of PDLF vitality, proliferation, enzyme activity and total protein synthesis including collagen *in vitro* (7, 8, 10, 17, 26). These favorable results probably occurred because milk contains important nutritional substances, such as amino acids, carbohydrates and vitamins. In addition, commercially available milk is pasteurized, which may inactivate enzymes that could be potentially harmful to the PDLF. The suitability of milk as a storage medium is further emphasized by its general availability.

ViaSpan has been shown to decrease the incidence of root resorption after replantation (27) and to be better than milk or HBSS for preserving the vitality of lip fibroblasts at room temperature. After 24 h of storage, the vitality of cells in milk, HBSS and in ViaSpan was 20.2%, 71.3% and 76.7%, respectively (17). These results are in contrast to the present study, in which PDLF stored in ViaSpan for 24 h had lower viability (10%–12%) and mitogenicity (37%–40%) when compared to HBSS or milk. This discrepancy might be attributable to the different experimental conditions (PDLF *vs* lip fibroblasts or 4°C *vs* room temperature) (25).

Interestingly, the clonogenic capacity of the PDLF stored in ViaSpan for up to 8 h was high and comparable to culture medium, α-MEM and HBSS, and statistically higher than milk. This ability dropped by 65% after 24 h when compared to the control group, and was lower by 45% when compared to milk or HBSS. It may be postulated that many of the surviving PDLF after storage of up to 8 h were progenitor cells. However, the sum of the low functional abilities of PDLF stored in ViaSpan when compared to other tested media, the high price (USD 300 per liter), the short shelf life (a few months), and the difficult access (only in pharmacies of selected hospitals), reduce its value as a storage medium.

Culture medium (Eagle's medium) at 37°C can preserve PDLF for extended periods (3, 14–16). In this study, PDLF stored in culture medium and α-MEM (4°C) had relatively high viability, mitogenic and clonogenic capacity up to 8 h of storage. Surprisingly, when the storage time exceeded 24 h, culture medium and α-MEM were less effective than milk or HBSS, which could be attributed to the low temperature (4°C) of storage in this study. This low tempera-

ture may have induced aggregation and thus lowered the cell's functional capacity. This is supported by our consistent finding that cells stored in α-MEM and culture medium for 24 h had a greater tendency to aggregate than cells stored in other media. These results were in agreement with a clinical study where teeth, after being stored in ice, showed extensive root resorption after replantation (28). In several studies, it has been suggested that soaking cold-stored tooth in warm saline before replantation may decrease this resorption (29–30). However, this pretreatment may be effective only in teeth with less than 15 min extra-oral time (31). Additional studies are needed to evaluate the effectiveness of culture medium and α-MEM at higher temperatures.

Conditioned medium was first introduced by Hupp et al. (19). The PDL of teeth stored for 96 hours in CM had more mitotic activity when compared to ViaSpan or HBSS. However, the ability of CM to preserve the vitality of PDLF in the present study was relatively low after 2, 8 and 24 h (97%, 93% and 91% of the control, respectively). CM induced the lowest mitogenicity after 2 and 24 h, 79% and 76% when compared to the control, respectively ($P<0.04$), and the lowest clonogenic capacity after 24 h. The higher mitogenic capacity of PDLF stored in CM after 8 h compared with 2 h can be explained by its non-standardized constitution, which is the main disadvantage of this storage medium. The stable growth factors in the CM during storage at −16°C before use is questionable and should be further investigated. The differences between the results of Hupp et al. (19) and those of the present study may be due to the different conditions: storage times (6, 48 and 96 h *vs* 2, 8 and 24 h), origin of the CM (from human gingival fibroblasts *vs* from HPDLF) and cell type (dog teeth *vs* HPDLF). The lower results of CM may be attributable to the non-effectiveness of growth factors after long storage in −16°C and to the deterioration effect of the waste released from cultured cells during the preparation of this media.

It can be concluded that the reduction in viability, when evaluated by trypan blue exclusion test, did not correlate with the clinical effectiveness of the storage medium. Mitogenicity and clonogenic capacity showed different achievements, but the clonogenic capacity test was the most sensitive assay to show statistically significant differences between various tested media and between different periods of incubation. Hank's balanced salt solution was the most effective storage medium for preserving the viability, mitogenicity and clonogenic capacity of PDLF after storage at 4°C up to 24 h followed by milk.

Acknowledgement – This study was supported by a grant from the Chief Scientist, Ministry of Health, Israel.

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