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In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in four media at room temperature

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Abstract – The choice of storage medium for preserving traumatically avulsed teeth is important for the success of future replantation. The objective of this study was to compare the effectiveness of four recommended storage media (Hank's balanced salt solution [HBSS], culture medium, α minimal essential medium [α-MEM], and ViaSpan) to preserve cultured periodontal ligament fibroblasts (PDLF) at room temperature (22°C). PDLF were obtained from explants of extracted healthy human teeth. Plates with confluent PDLF were soaked in the various media for 2, 8 and 24 h at room temperature. A control group was incubated with culture medium at 37°C. After incubation, viability of the cells was determined by trypan blue exclusion test. Viable cells were then analyzed for mitogenic (with thymidine) and clonogenic capacity (by culturing one cell/well). Viability of PDLF stored up to 24 h was comparable in all tested media, and the differences were limited to 1%–3%. PDLF stored for up to 24 h in various media had statistically comparable mitogenicity to the control group. After 8 h of storage, the differences were limited to 2%–9%, except for the α-MEM group which had 23%–29% lower mitogenic capacity compared to the control group. Increasing the storage time up to 24 h further decreased the mitogenicity of the cells by 22%–47%. The highest mitogenicity after 24 h of storage was found in PDLF stored in culture medium or HBSS, and the lowest in α-MEM. PDLF stored for 2–8 h in various media had a comparable clonogenic capacity to the control group. However, after 24 h, the cells' clonogenic ability dropped by 14%–66%. A similar trend of reduction was noted in the mitogenic and clonogenic capacity, although it was statistically significant only in the clonogenic capacity. Culture medium and ViaSpan, followed by HBSS, were the most effective in preserving the clonogenic capacity of PDLF after 24 h of storage. The lowest clonogenic capacity after 24 h of storage was in the α-MEM group (66%, $P<0.0025$). In conclusion, culture medium, followed by HBSS and ViaSpan, was the most effective media for preserving the viability, mitogenicity and clonogenic capacity of PDLF stored for up to 24 h at room temperature. The lowest functional abilities were found in PDLF stored in α-MEM.

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

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Tooth avulsions constitute 0.5%–16% of all traumatic injuries to permanent anterior teeth (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 a suitable 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 for quickly repopulating the denuded root surface with PDLF and preventing the osteoclasts from attaching to the cementum.

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 short periods (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). In recent years, Hupp et al. (11) introduced conditioned medium (CM) as a storage medium for avulsed teeth. It is the supernate of nonconfluent 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 because of the presence of growth factors released to the supernate during the exponential growth of the fibroblasts. However, in a previous study, Ashkenazi et al. (12) have shown that PDLF stored for 24 h in CM had the lowest viability, mitogenicity and clonogenic capacity when compared to PDLF stored in Hank's balanced salt solution (HBSS), milk, culture medium, α-MEM or ViaSpan. The main disadvantage of this medium is its non-standardized constitution. In addition, the stability of its growth factors during storage at -16°C before use is questionable.

Another currently recommended storage medium for avulsed teeth is milk, which has a relatively physiologic osmolarity with low bacterial content compared to saliva (6, 8, 9, 13–15). Milk has been shown to be effective as a storage medium for up to 12 h (13). In a previous study, Ashkenazi et al. (12) have shown that HBSS and milk are the most effective storage media for preserving the viability, mitogenicity and clonogenic capacity of PDLF after storage of up to 24 h. However, its effectiveness depends on low-temperature storage to prevent souring of the milk. The effect of low-temperature storage on the clinical success of replantation was studied by Andreasen et al. (16). They showed that teeth, after being stored in ice, showed extensive root resorption after replantation. Several authors suggest soaking the cold stored tooth in warm saline before replantation to decrease this resorption (17, 18). However, this pretreatment may be effective only in teeth with less than 15 min extra-oral time (19). These studies may support storage of avulsed teeth in media at room temperature, especially for extended periods.

Currently, there are four recommended media for preserving avulsed teeth for extended periods of time at room temperature. HBSS, the most popular and a very promising storage medium, it 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 cultured PDLF and avulsed teeth for extended periods of time (20, 21, 11, 12). The second medium is ViaSpan, which is an extremely effective cold storage medium for organs before transplantation (22). 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 (20). HBSS and ViaSpan were extremely effective for the first 24 h. Other recommended storage media include fibroblast culture media. In the 1970s several studies showed that culture medium (Eagle's medium at 37°C) can preserve PDLF for extended periods before replantation (3, 23–25). Viable cells can be trypsinized and cultured from teeth preserved in culture medium (37°C) as long as 1 year after extraction (23).

Among the currently recommended storage media, there are differences in the availability, price and long-term stability, nevertheless, there are only a few studies that compare *in vitro* the effectiveness of these storage media in preserving PDLF. Various *in vitro* assays have suggested evaluating the effectiveness of the storage media. 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 (11, 26). Recently, it was 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, 14).

After a study of the effect of cold storage (4°C) (12) on the functional abilities of PDLF, the purpose of this *in vitro* study was to compare the viability, mitogenicity and clonogenic capacity of PDLF stored in four different storage media, for 2, 8 and 24 h at room temperature (22°C).

Material and methods

General procedure

Cultured human PDLF 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 out, and 4 mL of the tested storage medium was added to each plate. The plates (two dishes in each group) were placed at room temperature (the room was air-conditioned and adjusted to 22°C) for 2, 8 and 24 h. Control dishes were incubated at 37°C in a humidified air containing 7% CO_2 with culture medium. After 2, 8 and 24 h, the medium was suctioned, the cells were washed (x3, 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 (27). 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 (28). PDLF 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-fold concentrated AB. 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 3–5 passages.

Storage media

The media examined were culture medium, α-MEM (Biological Industries Co, Beit Haemek, Israel), HBSS (Biological Industries) and ViaSpan (No. 1000–16–06, Dupont, Belzer UW, Israel). The culture medium was composed of α-MEM supplemented with 15% FCS (Biological Industries) and the antibiotic solution (AB) of penicillin (100 units/mL), gentamicin (50 μg/mL) and fungizone (0.3 μg/mL) (Biological Industries). HBSS was composed of 8 g/L sodium chloride, 0.4 g/L D-glucose, 0.4 g/L potassium chloride, 0.35 g/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).

Mitogenic assay

Mitogenic capacity of the cells represents the functional ability of all the cell population (not just the progenitor cells) to attach to a solid surface and proliferate. After incubation with the examined medium, cells from each plate were washed (PBS ×3), 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), cells were rinsed (x3, 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 three times. In each experiment, two plates (12 wells) of cultured cells were used for each tested medium.

Clonogenic capacity

The clonogenic capacity of PDLF estimates the proportion of progenitor cells in a population with extensive proliferative and colony-forming capacities (14, 15). This may represent the effectiveness of each medium to preserve the progenitor cells. To evaluate the clonogenic capacity of the stored cells, cells from each tested group were inoculated into two 96-well plates at a concentration of one viable cell/well. The cells 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 covering 75% 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 were repeated three times (six 96-well plates for each tested group).

Statistical analysis

Differences among the various media (HBSS, culture medium, α-MEM 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

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

	Incubation time		
	2 h	8 h	24 h
Control	93.4±1.5	93.4±1.5	93.4±1.5
HBSS	97.5±2.5	94.0±0.7	92.3±1.8
Culture medium	92.3±4.8	90.7±1.8	94.3±1.1
α-MEM	97.5±2.5	94.7±3.6	92.3±3.6
ViaSpan	97.5±2.5	91.7±6.4	94.7±0.9

Note: Results represent the mean ±SD of three experiments (6 different plates). No significant differences were found between different times of incubation (2 h, 8 h or 24 h), or between the various experimental groups.

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±51	100±51	100±51
HBSS	94±33	98±28	77±37
Culture medium	106±70	91±21	78±59
α-MEM	71±37	77±8	53±29
ViaSpan	103±54	97±7	56±41

Note: Results represent the mean±SD of three experiments. In each experiment 12 wells were evaluated. No significant differences were found between different times of incubation (2 h, 8 h or 24 h), or between the various experimental groups.

(χ^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. Viability of PDLF stored up to 24 h at room temperature was comparable in all 4 tested media, and the differences were limited to 1%–3% only.

The results of the mitogenic capacity of the cells are summarized in Table 2. PDLF stored for up to 24 h had statistically comparable mitogenic capacity compared to the control group and to their mitogenic capacity after 2 or 8 h of storage. However, the differences in the mitogenic capacity (as a percentage) between groups were higher than in the viability test especially after 24 h of storage (Fig. 1–3). After 2–8 h of storage, the mitogenic capacity of the PDLF showed only 2%–9% reduction compared to the control group, except for cells stored in α-MEM, which had the lowest proliferative ability compared to other tested media (−29%, P=0.1) (Fig. 1 and 2). However, after 24 h of incubation, the mitogenic capacity of the cells dropped by 22%–47% when compared to the control group (culture medium, α-MEM and ViaSpan, P=0.1) and by 18%–46% when compared to their mitogenic capacity after 2 h of storage (α-MEM P=0.2, culture medium P=0.2, ViaSpan P=0.1). The lowest proliferative ability after 24 h was found in cells stored in α-MEM (53%) or ViaSpan (56%) (P=0.1).

The clonogenic capacity of PDLF after storage in four media for 2, 8, and 24 h is presented in Table 3. Up to 8 h of storage, clonogenic capacity was similar in all four tested media, and the differences between groups were not statistically significant. After 2 h of storage the clonogenic capacity dropped by 7%–32% (P<0.3). After 8 h of storage, the clonogenic capacity of stored PDLF increased in all groups by 4%–41% as compared to the control group. This increase was not of statistical significance in any group. These re-

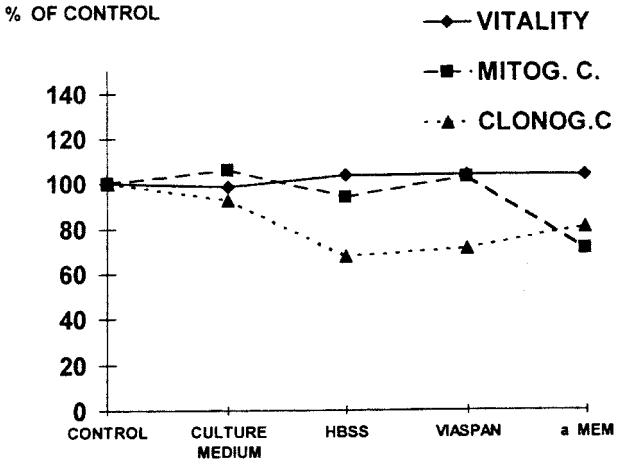


Fig. 1. Functional abilities of PDLF after 2 h of storage in various media.

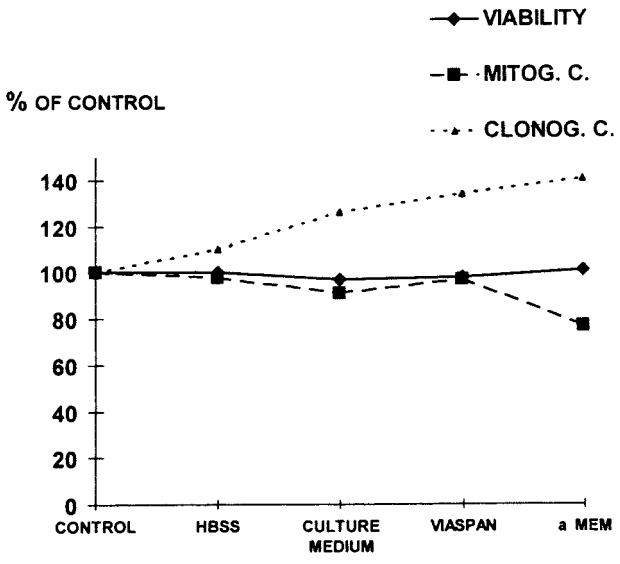


Fig. 2. Functional abilities of PDLF after 8 h of storage in various media.

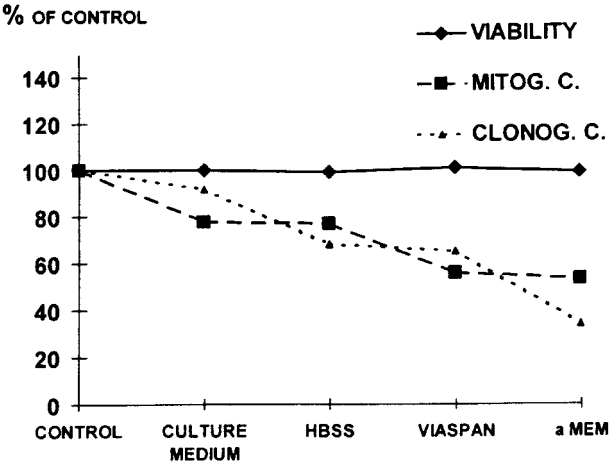


Fig. 3. Functional abilities of PDLF after 24 h of storage in various media.

sults were in contrast to the mildly decreased activity in the mitogenic capacity. After 24 h of storage, the clonogenic capacity of the cells dropped by 14%–66% when compared to the control group (Fig. 3). Although a similar trend of reduction was noted in the mitogenic capacity assay, the reduction was statistically significant only in the clonogenic capacity. Significant differences were found in the α-MEM and HBSS groups ($P<0.0025$ and $P<0.01$, respectively). Cells stored in culture medium had only 14% reduction in their clonogenic capacity after 24 h of storage as compared to the control group ($P=0.5$), and higher clonogenic capacity when compared to α-MEM, HBSS and ViaSpan ($P=0.07$, $P=0.4$ and $P=0.16$, respectively).

Discussion

Experimental design

This study was conducted in order to compare the effectiveness of four of the best storage media to preserve the functional abilities of periodontal ligament fibroblasts. PDLF 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. In our previous study we evaluated the viability, mitogenicity and clonogenic capacity of PDLF stored at 4°C (12). In the present study, we evaluated the same functional abilities at room temperature, in order to evaluate the effect of different storage temperatures (4°C *vs* 22°C) on cell function. It was assumed that room temperature might increase cell function (12). The possible deteriorating effect of storage in cold temperature was studied by Andreasen et al. (16). They showed that teeth stored in ice showed extensive root resorption after replantation (16). This experimental design ruled out the use of milk as a storage medium, although cold milk is well known as an effective storage medium. Stored at room temperature for as long as 24 h, milk will sour.

Functional abilities

The effectiveness of the tested media was evaluated not just by the vitality, but also by the ability of the cells to function, as manifested by the mitogenic and clonogenic capacities of the PDLF after storage. In this study, the functional abilities were not preserved similarly. Thus, a medium that induced high viability or mitogenic capacity could induce low clonogenic capacity (Fig. 1–3). Nevertheless, the optimal medium should preserve most of the functional capabilities. The importance of one function over another in predicting clinical success is not known.

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 method is quick, easily performed and differentiates clearly between dead and viable cells. Cell recovery after storage in almost all of the experimental conditions was very high (92%–98%). In a similar study the viability of PDLF after 24 h of storage at 4°C was 82%–97%, depending on the various storage media (12). Thus, the viability of PDLF stored for up to 24 h at room temperature was higher by 0%–15% than when stored at 4°C. The most noticeable difference was found in the ViaSpan group, in which the viability of PDLF stored at room temperature was higher by 15% than when stored at 4°C.

Hiltz & Trope (20) found that the viability of lip fibroblasts after 24 h of storage in HBSS and ViaSpan at room temperature was 71.3% and 76.7%, respectively. The differences between these studies can be

Table 3. Percentage of cells with high clonogenic capacity (75% to confluent) after 2, 8 and 24 h of incubation in different media

	Incubation time		
	2 h	8 h	24 h
Control	10.8±8.7	10.8±8.7	10.8±8.7
HBSS	7.3±0.4	11.2±3.9	7.3±6.1[a,c]
Culture medium	10.0±1.3	12.5±5.4	9.3±0.6
α-MEM	8.7±3.8	15.2±9.2[b]	3.7±2.5[a,b,c]
ViaSpan	7.7±3.5	14.5±7.7	7.0±4.6[c]

Note: The results represent the mean±SD of three experiments (576 cells for each group).
[a]Significant differences when compared with the control.
[b]Significant differences when compared with 2 h of incubation.
[c] Significant differences when compared with 8 h of incubation.

attributed to the origin of the cells, i.e., lip fibroblasts *vs* PDL explants from very young adults and early passage (3–6, 29). Nevertheless, this absolute *in vitro* high recovery showed a weak correlation with the low clinical success rate, which is in agreement with others (5, 26).

Mitogenic capacity

In this study, PDLF stored for 2–8 h in the various media had similar mitogenic capacity when compared to the control. However, after 24 h the differences between control and experimental groups were increased by 22%–47%, although these differences were not of statistical significance ($P=0.1$). These results were in agreement with previous studies in which the proliferative capacity of PDLF decreased with longer storage times (11, 30). The relatively high mitogenicity of cells after 2–8 h could be explained by the pre-selection of only the best, well-known storage media. The lack of significant differences after 24 h may be attributed mainly to the larger variability in the mitogenic capacity assay in general, though a trend could be noticed.

In our previous study the mitogenicity of PDLF was evaluated after storage at 4°C (12). The effect of temperature (22°C *vs* 4°C) on mitogenic capacity was evaluated by comparing the mitogenic capacity of PDLF with the control group in each experiment. This comparison showed that generally, the mitogenic capacity of PDLF was higher when stored at 4°C as compared to room temperature (Table 4). For instance, the mitogenicity of PDLF stored in HBSS, culture medium, α-MEM and ViaSpan for 2 h at 4°C was higher by 15%, 44%, 59% and 7%, respectively. The higher mitogenic capacity at 4°C than at room temperature may be attributed to the priming effect of the low temperature on the stored cells.

Table 4. The effect of storage temperature (4°C *vs* room temperature) in various media on the mitogenic capacity of PDLF

Storage time	2 h		8 h		24 h	
Storage temperature	4°C	Room temperature	4°C	Room temperature	4°C	Room temperature
Control	100	100	100	100	100	100
HBSS	109	94	107	98	102	77
Culture medium	150	106	98	91	84	78
α-MEM	130	71	95	77	81	53
ViaSpan	110	103	90	97	61	56

Note: The values represent % of CPM (counts of radiation per min) compared with control.

Table 5. The effect of storage temperature (4° *vs* room temperature) in various media on the clonogenic capacity of PDLF

Storage time	2 h		8 h		24 h	
Storage temperature	4°C	Room temperature	4°C	Room temperature	4°C	Room temperature
Control	100	100	100	100	100	100
HBSS	103	68	95	110	62	68
Culture medium	106	93	97	126	46	92
α-MEM	149	81	100	141	35	34
ViaSpan	108	71	116	134	35	65

Note: The values represent % of clonogenic capacity compared with control.

Clonogenic capacity

This study indicated that the clonogenic capacity of the cells was statistically comparable to the control after up to 8 h of storage. However, longer storage time (24 h) significantly decreased this ability. Interestingly, the clonogenic capacity of the PDLF stored in the tested media was highest after 8 h of storage. This ability dropped by 26%–76% after 24 h when compared to their ability after 8 hours and was lower by 14%–66% compared to the control group. It may be that the contribution of the priming effect, which triggers the cells to proliferate, was more dominant than the deterioration effect of RT after up to 8 h of storage. However, at longer storage time (24 h) the deterioration effect of room temperature was more pronounced.

The effect of storage temperature (22°C *vs* 4°C) on clonogenic capacity was evaluated by comparing the clonogenic capacity of stored PDLF with the control group in each experiment (12) (Table 5). This comparison indicated that the effect of storage temperature on clonogenic capacity depends on the duration of storage. At 4°C, the highest values of clonogenic capacity were observed after 2 h of storage, while at room temperature the highest values of clonogenic capacity were observed after 8 h of storage.

After 2 h of storage the clonogenic capacity of PDLF stored at 4°C was higher than at room temperature. However, after 8 h and 24 h of storage, PDLF stored at room temperature had 10%–86% higher clonogenic capacity than PDLF stored at 4°C, possibly because the progenitor cells were more sensitive to low-temperature storage than the whole cell population.

Storage media

Culture medium (Eagle's medium) at 37°C can preserve PDLF for extended periods (3, 23–25). In this study culture medium (including all additives: FCS, AB and ribonucleotides) was the most effective medium for preserving the viability, mitogenicity and clonogenic capacity of PDLF up to 24 h at room temperature as compared to HBSS, ViaSpan or α-MEM.

HBSS is a popular storage medium that has been shown to be effective in preserving avulsed teeth for extended periods of time (11, 20, 21). In this study, HBSS was the second best medium to preserve the viability, mitogenic and clonogenic capacity of cells after storage of up to 24 h at 22°C.

ViaSpan was shown to decrease the incidence of root resorption after replantation (31) 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 HBSS and in ViaSpan was reported to be 71.3% and 76.7%, respectively (20). These results were in agreement with our previous results in which the viability of PDLF stored for 24 h in HBSS and ViaSpan was comparable. However, our absolute numbers were higher, which might be explained by the different origin of the cells, i.e., explants from very young adults and early passage (3–6, 29).

The sum of the functional abilities of PDLF stored in ViaSpan was comparable to HBSS but lower than cultured medium. However, its high price (USD 300 per liter), the short shelf-life (a few months), and the difficult access (only in pharmacies of selected hospitals), make culture medium and even HBSS better for storage of avulsed teeth up to 24 h at room temperature from the practical aspect.

It can be concluded that the reduction in the viability, when evaluated by trypan blue exclusion test, does not correlate with the clinical effectiveness of the storage medium. Mitogenicity showed notable but not statistically significant reduction after 24 h of storage at room temperature. Clonogenic capacity showed similar but not identical results. After 24 h some storage media showed statistically lower ability than control. Culture medium, followed by HBSS and ViaSpan, the most effective storage media for preserving the viability, mitogenicity and clonogenic capacity of PDLF after storage of up to 24 h at 22°C, whereas α-MEM was the least effective medium for preserving these functional abilities.

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