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In vitro viability, mitogenicity and clonogenic capacities of periodontal ligament fibroblasts after storage in four media supplemented with growth factors

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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 evaluate the effectiveness of growth factors (IGF-1 and PDGF-BB) when added to storage media in preserving the functional abilities of cultured periodontal ligament fibroblasts (PDLF). The evaluated storage media were: ViaSpan, Hanks' balanced salt solution (HBSS), α minimal essential medium (α MEM), and α MEM supplemented with FCS and antibiotic (α MEM-S). PDLF were obtained from explants of human healthy extracted teeth. Plates with confluent PDLF were soaked in the various media supplemented with IGF-1 (10 ng/ml) and PDGF-BB (4 ng/ml) for 2, 8 and 24 h at room temperature (24°C). The control group was incubated with the examined storage media without growth factors at 24°C. An additional control group was incubated with culture medium at 37°C without growth factors. After incubation, the viability of the cells was determined by Trypan blue exclusion test. Viable cells were then analyzed for mitogenic (with thymidine) and clonogenic (by culturing one cell/well) capacities. Storage of PDLF with growth factors (GF) for 2, 8 and 24 h decreased their vitality by only 3% (not statistically significant). The mitogenicity of PDLF stored for 2, 8 and 24 h in various media with GF was statistically comparable to that of the control group. Generally, the highest mitogenic capacity of PDLF stored with or without GF was found after 8 h of storage. Increasing the storage period to 24 h decreased the mitogenic capacity of the cells stored with GF by only 10–40% compared to the control group. In contrast, the clonogenic capacity of PDLF stored with GF increased with increasing storage periods by 100–300%, and the highest clonogenic capacity was found in most storage media after 24 h of storage with GF. The highest clonogenic and mitogenic capacities were found in cells stored in HBSS followed by α MEM-S. The mitogenic and clonogenic capacities of PDLF stored in various media supplemented with GF for 2–8 h were generally lower than without GF supplementation. The mitogenic and clonogenic effects of GF-supplementation was observed only after 24 h of stor-

age. After 24 h of storage with GF, the clonogenic capacity increased by 8–224% and the mitogenicity by 20–37%, except in cells stored in α MEM (-1%). However, these differences were generally not statistically significant. In conclusion, the mitogenic and clonogenic effects of GF were observed only after 24 h of storage at room temperature. HBSS and α MEM-S supplemented with GF were the most effective media for preserving the viability, mitogenicity and clonogenic capacity of PDLF stored for 24 h at room temperature. For short periods of storage (2 and 8 h), HBSS and α MEM-S without GF were preferable.

Key words: growth factors; clonogenic capacity; mitogenic capacity; storage medium; tooth-avulsion; PDL fibroblasts

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Successful replantation of an avulsed tooth depends on the presence of viable periodontal ligament cells, capable of proliferating over the denuded damaged root surface (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 and the functional capabilities of the injured PDL cells and their progenitors. This is essential for swift repopulation of the denuded root surface with PDL cells and to prevent the osteoclasts from attaching to this area.

Recently, four media have been shown to preserve avulsed teeth for extended periods at room temperature (24 °C) (7): Hanks' Balanced Salt Solution (HBSS), ViaSpan, and fibroblast culture media (Eagle's medium, α-MEM, α-MEM S).

HBSS, the most popular and a very promising storage medium, has a pH of approximately 7 and an osmolarity of 270–290 mOsm/l. This medium has been shown to effectively preserve cultured PDLF and avulsed teeth for extended periods of time (8–11).

ViaSpan is an extremely effective cold storage medium for organs before transplantation (12). Its osmolarity is 320 mOsm/l, with a pH of approximately 7.4, which is ideal for cell growth. ViaSpan was shown to be extremely effective in preserving the viability of lip fibroblasts for the first 24 h (10).

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, 13–15). Viable cells can be trypsinized and cultured from teeth preserved in culture medium (37 °C) as late as 1 year after extraction (13).

The extra alveolar period might be utilized not just to preserve the PDL cells by using optimal storage medium, but also to stimulate their growth by adding growth factors to the storage medium. Polypeptide growth factors (GF) are a class of natural biological mediators that regulate the proliferation, differentiation, motility and matrix synthesis of nearly all cell types (16). Two of the best characterized factors, platelets derived growth factor (PDGF) (A and B) and insulin growth factor (IGF), interact synergistically to accelerate the healing of partial thickness skin wounds and enhance the regeneration of periodontal tissues (17, 18). Topical application of PDGF and IGF stimulates DNA, collagen and noncollagenous protein synthesis, resulting in doubling connective tissue volume within the wound during the 1st week of healing. Lynch et al. (19) demonstrated that short-term application of a combination of PDGF-B and IGF can enhance the formation of periodontal attachment apparatus 5–10 fold during the early phases of wound healing following surgery. In addition, a physiologic periodontal ligament space was formed between the new bone and new cementum, with no increase in ankylosis in the treated sites. Recent studies by Matsuda et al. (20) and Oates et al. (21) indicate that PDGFs are potent mitogenic agents for PDLF *in vitro*. The exact mechanisms by which PDGF/IGF enhance periodontal regeneration remain to be fully elucidated. The American Academy of Periodontology's position paper on growth factors (22) stated that these polypeptides growth factors possess a greater ability to control cell growth and differentiation than any other class of molecules currently used in dentistry.

To utilize the effect of growth factors, Hupp et al. (8) introduced conditioned medium (CM) as a storage medium. CM is the supernate of non-confluent PDLF harvested after 2 days of culturing. The idea was that this storage medium would not only preserve the viability of PDLF of avulsed teeth, but would also stimulate their growth by the presence of growth factors released to the supernate during the exponential growth of the fibroblasts. However, in a previous study, Ashkenazi et al. (9) have shown that PDLF stored for 24 h in CM had the lowest viability, mitogenicity and clonogenic capacity compared to PDLF stored in HBSS, milk, culture medium, α MEM or ViaSpan. The main disadvantage of this medium is its non-standardized constitution.

Various *in vitro* assays have been suggested for evaluating the effectiveness of storage media. Previous reports indicate a weak relationship between various *in vitro* measures of cell viability and clinical success rates (8, 23). Recently, it was shown that *in vitro* assays of clonogenic capacity are much more sensitive to

extraoral storage time and storage conditions than dye inclusion or cell attachment (7, 9, 24).

The purpose of this *in vitro* study was to evaluate the effectiveness of growth factors in preserving the viability, mitogenicity and clonogenic capacity of PDLF stored in four storage media at non-optimal condition (room temperature, 24 °C).

Material and methods

General procedure

Cultured human PDL fibroblasts were placed in 30-mm petri dishes and grown to confluence 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 supplemented with IGF-1 (10 ng/ml) and PDGF-BB (4 ng/ml) 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 24 °C) for 2, 8 and 24 h. To evaluate the effect of GF, a control group containing storage medium without growth factors was added to different plates and incubated at room temperature. To compare these data with undisturbed cells, under natural conditions, additional control dishes were incubated at 37 °C in a humidified air containing 7% CO_2 with culture medium without growth factors. After 2, 8 and 24 h, the medium was suctioned, the cells were washed (×3) with phosphate buffered saline (PBS) containing Ca^{++} Mg^{++} 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 resuspended in 2 ml of fresh culture medium. The cells were then evaluated for their viability, mitogenic and clonogenic capacities.

Periodontal ligament fibroblasts

Cultures of human PDL fibroblasts were initiated by explantation and maintained in our laboratory as previously described (7, 9, 26).

Storage media

The media examined were ViaSpan (No. 1000–16–06, Dupont, Belzer UW), HBSS (Biological Industries), α-MEM (Biological Industries, Beit Haemek, Israel) and α-MEM-S {(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)}.

Growth factors

The growth factors used were human recombinant platelet derived growth factor BB (PDGF-BB, Cat No. D-3201 Sigma, St. Louis, MO, USA) and insulin growth factor-1 recombinant analog (IGF-1, Cat No. I-1271, Sigma). In a preliminary study, the maximum mitogenic effect of IGF-1 and PDGF-BB was observed at a concentration of 10 ng/ml and 4 ng/ml, respectively. IGF-1 powder containing 100 μg IGF-1 was reconstituted in 400 μl of 0.1% BSA (Cat No. A-4503, Sigma) and in 100 μl of 10 mM HCl. Aliquots of stock solution were stored at −20 °C at a concentration of 200 μg/ml. At the start of the experiment, 6 μl of the stock solution were diluted with 30 ml of storage media to get stock solution in a concentration of 40 ng/ml. This storage media was again diluted 1:4 with storage media to get a final concentration of 10 ng/ml.

PDGF-BB was obtained by reconstituting the content of the vial (10 μg powder) with 1 ml of 4 mM HCl containing 0.1% BSA. Vials containing stock PDGF-BB in a concentration of 10 μg/1 ml were stored in working aliquots at–20 °C until use. At the start of the experiment, 11 μl of stock solution was added to 27.5 ml of storage media containing IGF-1 (at concentration of 10 ng/ml) to get the final concentration of 4 ng/ml.

Viability

The stored cells were counted in a hemocytometer and the viability was determined by Trypan blue exclusion test (25). 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 capacities as described below.

Mitogenic assay

The mitogenic capacity of the cells represents the functional ability of the entire cell population (not just the progenitor cells) to attach to a solid surface and proliferate. After incubation with the examined medium, mitogenic assay was performed using H^3 thymidine as previously described (7, 9). 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 (24, 27). This may suggest the effectiveness of each medium to preserve the progenitor cells. Stored cells

were evaluated for their clonogenic capacity by plating one cell/well, as previously described (7, 9). 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 medium tested).

Statistical analysis

Differences among the various media (HBSS, α-MEM-S, 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 (χ^2) and the differences in the radioactive thymidine incorporation (mitogenic capacity) were evaluated using the Wilcoxon rank sum test.

Results

Viability

The results of the vitality tests of cells stored with various media supplemented with growth factors are summarized in Table 1. Storage of PDLF for 2, 8 and 24 h decreased their vitality by only 3%. This decrease was not statistically significant. Vitality of the PDLF after 24 h was comparable in all 4 tested media (Figs. 1–3). The highest viability after 24 h of storage was found in cells stored in α-MEM-S and ViaSpan. The supplementation of GF to the storage media had no significant effect on the viability and the differences ranged from +4.7% to −3.0%.

Table 1. Percent viability of PDLF after incubation in various storage media with or without supplementation of growth factors at room temperature

	Time of incubation		
	2 h	8 h	24 h
Control (37°C)	93.4±1.5	93.4±1.5	93.4±1.5
HBSS with GF	95.5±0.5	92.7±3.1	91.7±1.8
HBSS without GF	96.5±2.5	94.0±0.7	92.3±1.8
Difference	−1%	−1.4%	−0.6%
α MEM-S with GF	94.5±1.5	95.0±0.7	93.0±4.7
α MEM-S without GF	92.3±4.8	90.7±1.8	94.3±1.1
Difference	+2.4%	+ 4.7%	−2.6%
α-MEM with GF	94.5±5.5	92.3±4.2	90.7±6.2
α-MEM without GF	97.5±2.5	94.7±3.6	92.3±3.6
Difference	−3%	−2.5%	−1.7%
ViaSpan with GF	98.0±2.0	96.0±3.3	93.3±3.6
ViaSpan without GF	97.5±2.5	91.7±6.4	94.7±0.9
Difference	+ 0.5%	+ 4.6%	−1.4%

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 among the various experimental groups.

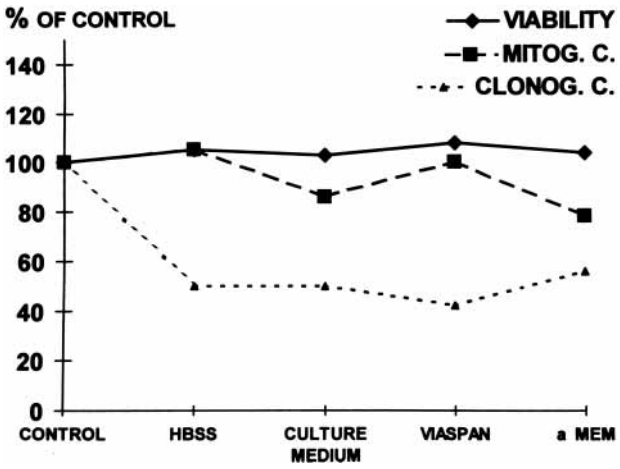


Fig. 1. Functional abilities of PDLF after 2 h of storage.

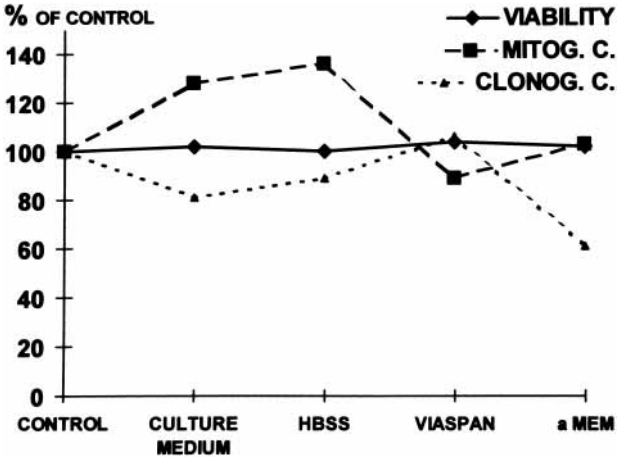


Fig. 2. Functional abilities of PDLF after 8 h of storage.

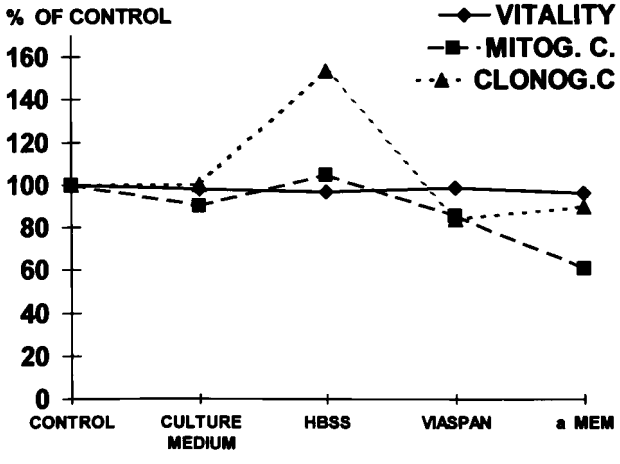


Fig. 3. Functional abilities of PDLF after 24 h of storage.

Mitogenic capacity

The results of the mitogenic capacity of the cells stored in various media supplemented with growth factors are summarized in Table 2. The mitogenicity of PDLF stored for 2, 8 and 24 h in various media with GF was statistically comparable to that of the control group. Generally, the highest mitogenic capacity was found after 8 h of storage and the lowest after 24 h of incubation (Figs. 2 and 3). The mitogenic capacity of PDLF after 24 h of storage was 10–40% lower compared to the mitogenicity after 8 h of storage. After 2 h of storage, the differences in mitogenicity between groups were limited to +5% to –22%, compared with controls ($P>0.5$). The lowest mitogenicity after 2 h storage was found in α MEM (–22% compared with control, $P>0.5$) (Fig. 1). The highest mitogenicity after 8 h and 24 h of storage was found in HBSS (136% and 104%, respectively, compared with controls) followed by α-MEM-S (128% and 90%, respectively). The lowest proliferative ability after 24 h was found in cells stored in α-MEM or ViaSpan: 60% and 85%, respectively ($P>0.5$). The mitogenic effect of GF was expressed only after 24 h of storage. PDLF stored for 24 h with GF showed 20–37% higher mitogenicity than cells stored without GF, except for the α-MEM group (–0.9% difference); however the differences were not statistically significant ($P>0.5$).

Clonogenic capacity

The clonogenic capacity of PDLF after storage in various media supplemented with growth factors for 2, 8 and 24 h is presented in Table 3. Generally, PDLF stored for 2 h and 8 h in various media with growth factors had a lower clonogenic capacity than in the control group (Figs. 1 and 2). Moreover, supplementation of growth factors to the media of cells stored for 2 and 8 h generally decreased the clonogenic capacity of stored cells by 5–54%. This decrease was not statistically significant except in α MEM after 2 and 8 h ($P<0.05$ and $P<0.0005$, respectively). After 2 h of storage the clonogenic capacity of stored cells was about 50% lower than that of the control group (HBSS $P=0.5$, α MEM $P=0.03$, α-MEM-S $P=0.7$, ViaSpan $P=0.15$) (Fig. 1). After 8 h of storage, the clonogenic capacity of stored PDLF increased in all groups by 9–154% as compared to their capacity after 2 h of storage (α MEM $P<0.008$). But the clonogenic capacity after 8 h of storage was lower than controls, except for the ViaSpan group, which had higher clonogenic capacity (6% higher than control, $P=0.2$). After 8 h of storage, significant differences were found between α-MEM-S and α MEM ($P=0.0007$), HBSS and α MEM ($P=0.0068$), α-MEM-S and ViaSpan ($P=0.06$). The clonogenic effect of growth factors supplemented to storage media was observed only after 24 h of storage. After 24 h, the clonogenic capacity of the PDLF increased in all groups by 24–71% compared to 8 h of storage (except for the ViaSpan group: HBSS $P=0.08$, α-MEM-S $P=0.08$, α MEM $P=0.0018$, ViaSpan $P=0.17$, compared to 8 h of storage) and by 100–300% compared to 2 h of storage (Fig. 3). Cells stored in HBSS for

Table 2. Mitogenic ability of PDLF following incubation in different media with or without supplementation of growth factors at room temperature

	Time of incubation		
	2 h	8 h	24 h
Control (37°)	100±51	100±51	100±51
HBSS with GF	105.3±46	136.0±7	103.7±85
HBSS without GF	116.0±33	129.7±28	82.7±37
Difference	−9.4%	+4.8%	+25.3%
α MEM-S with GF	85.7±49	127.7±21	90.3±72
α MEM-S without GF	93.3±70	120.0±21	75.0±59
Difference	−8.1%	+5.8%	+20.4%
α-MEM with GF	78.3±28	103.3±32	59.7±15
α-MEM without GF	79.7±37	104.3±8	60.3±15
Difference	−1.7%	−1.0%	−0.9%
ViaSpan with GF	99.7±55	88.7±19	85±37
ViaSpan without GF	101.7±54	125.7±7	62±41
Difference	−1.9%	− 29.4%	+37%

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

Table 3. Percentage of cells with high clonization capacity (75% to confluence) Results after 2, 8 and 24 h of incubation in different media with or without supplementation of growth factors at room temperature

	Time of incubation		
	2 h	8 h	24 h
Control (37°)	12.0±8.7	12.0±8.7	12.0±8.7
HBSS with GF	6.0±0.7	10.7±0.9	18.3±11.8[a]
HBSS without GF	7.3±0.4	11.3±0.4	14.3±8.4
Difference	−17.8%	−5.3%	+27.9%
α MEM-S with GF	6.0±1.3	9.7±2.9	12±4.7
α MEM-S without GF	10.0±1.3	13.0±2.7	9.3±0.4
Difference	−40%	−25.3%	+29%
α-MEM with GF	6.7±2.4[a,c]	7.3±4.9[b2,c]	10.7±10.2[a,b8,c]
α-MEM without GF	8.7±2.9	15.7±6.9	3.3±2.2
Difference	−22.9%	−53.5%	+224.2%
ViaSpan with GF	5.0±2.7	12.7±3.1	10.0±2.7
ViaSpan without GF	7.7±2.4	14.7±6.2	9.3±1.1
Difference	−35%	−13.6%	+7.5%

Results represent the mean±SD of three experiments (576 cells for each group).
[a]Significant differences when compared with the control.
[b]Significant differences among different periods of incubation (2 h, 8 h).
[c]Significant differences when compared with cells stored without growth factors.

24 h with GF had 53% higher clonogenic capacity compared with the control group ($P<0.0006$). Cells stored in α-MEM-S had the same clonogenic capacity as controls ($P=0.89$). The clonogenic capacity of PDLF stored in α MEM and ViaSpan for 24 h was only 11–17% lower than the control group (α MEM $P<0.005$, ViaSpan $P=0.24$). The clonogenic capacity of cells after 24 h storage with growth factors increased by 7.5–224%, compared with cells stored without growth factors (α MEM $P=0.00003$, α-MEM-S $P=0.39$, HBSS $P=0.4$ ViaSpan $P=0.73$), even though the storage was under non optimal conditions, such as storage at room temperature and room air.

Discussion

Experimental design

This study was conducted in order to evaluate the effect of growth factor supplementation on the functional abilities of periodontal ligament fibroblasts stored in four recommended storage media. The exact nature of the PDL cells responsible for repopulating the damaged root surface has not yet been clarified. However, fibroblasts are the most dominant, as well as the most easily cultured, cells in the PDL. These cells were therefore used in this study as well as others. 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 thought to be clinically relevant. IGF and PDGF growth factors were used in this study. These GF were shown to be potent mitogenic agents for PDLF *in vitro* (20, 21), and possess a greater ability to control cell growth and differentiation than any other class of molecules currently used in dentistry (22). The temperature of storage (room temperature) was found to be clinically relevant, and better than storage at 4 °C. Clinical studies showed that teeth, after being stored in ice, showed extensive root resorption after replantation (29, 30). In a previous *in vitro* study, storage at room temperature preserved the viability and the clonogenic capacity of PDLF better than storage at 4 °C after 8 and 24 h (7).

Functional abilities

The effectiveness of the tested media was evaluated by the viability and functional ability of the cells as manifested by the mitogenic and clonogenic capacities. In this study, there was no similarity between these functional abilities (Figs. 1–3). Thus, a medium that induced the highest mitogenic capacity after 8 h of storage could induce the highest clonogenic capacity after 24 h of storage. 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 clinical healing of replanted exarticulated teeth. In this study, the Trypan blue exclusion staining technique was used. This is a quick and easily performed technique which differentiates distinctly between dead and viable cells. Cell recovery after storage in almost all of the experimental conditions was very high (92–98% viability). In a similar study, the viability of PDLF after 24 h of storage at room temperature without supplementation of growth factors was higher by 0.6–1.6%, depending on the various storage media (9). However, these differences were not statistically significant and are in standard deviation range. Hiltz & Trope (10) 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 attributed to the origin of the cells, i.e. lip fibroblasts vs explants of PDLF from very young adults and early passage (3–6, 31). Nevertheless, this absolute high recovery showed a weak relationship to the low clinical success rate, which is in agreement with others (5, 23).

Mitogenic capacity

In this study, GF did not increase the mitogenicity of PDLF stored at room temperature for 2 and 8 h in the various media; in contrast, the mitogenicity decreased by 1–29% in most groups. However, after 24 h of storage, growth factors increased the mitogenicity by 20–37% ($P=0.4$). The net mitogenic effect of the growth factors in our experiment was relatively low. Clinical and laboratory studies, which used ideal conditions for cell-proliferation, have usually evaluated the mitogenic effect of growth factors for only days to 2 weeks after exposing the cells to the growth factors (18, 32–36). Oates et al. (36) in an *in vitro* study, evaluated the mitogenic response of human PDLF to PDGF BB and showed that after 24 h of incubation with 10 ng/ml PDGF BB there was about a twofold increase in thymidine incorporation. Wang et al. (34) showed in an *in vivo* study that 1 day after placing PDGF to fenestration defects in the root surface of posterior teeth in dogs, fibroblast labeled cells increased by 83%. The discrepancy between the above studies and this study (80–100% vs 20–37% increase, respectively) can be attributed to the non optimal conditions of this study (i.e. room temperature vs 37 °C, room vs incubator or *in vivo* and the use of various storage media vs culture medium or *in vivo*), which were used in an attempt to imitate the realistic conditions of an

avulsed tooth. Importantly, PDLF that were stored for 24 h in α-MEM-S or HBSS supplemented with growth factors had 90–104% mitogenic capacity compared with control cells. Thus, the growth factors actually diminished the deterioration during long storage at room temperature. Moreover, the net effect of growth factors on the repopulation of denuded areas of avulsed teeth can be improved and should be evaluated in *in vivo* studies. The decrease in the mitogenicity with time (8 h vs 24 h) is in agreement with previous studies in which the proliferative capacity of PDLF decreased with longer storage periods (11, 30). The relatively high mitogenicity of cells after 8 h could be explained by the priming effect on the stored cells, the mitogenic effect of the GF and the use of the most well-known storage media. The deterioration effect of a prolonged storage period decreased the mitogenicity after 24 h of storage. According to this study, in non optimal conditions, (i.e. room temperature, room air) a minimum of 24 h of exposure to GF is needed to increase the mitogenic capacity of stored cells. Interestingly, Ashkenazi et al. (9) evaluated the effect of conditioned medium on the mitogenic capacity of PDLF stored at 4 °C. They found that conditioned medium induced lower mitogenicity than HBSS, α MEM-S, milk and ViaSpan, and the highest mitogenic effect was observed after 8 h of storage.

Clonogenic capacity

The clonogenic capacity of cells stored up to 8 h in various media supplemented with GF was generally decreased when compared to storage without GF. However, the clonogenic capacity of the cells increased with increasing storage periods (after 8 h of storage, the clonogenic capacity increased by 9–154%, and after 24 h, by a further 24–75% in all groups except the ViaSpan group). This is in contrast to PDLF stored without GF, in which the clonogenic capacity was highest after 8 h of storage and decreased by 26–76% after 24 h (7). After 24 h of storage with GF, the clonogenic capacity of stored PDLF was not just level with that of cells stored without GF but was even 7.5–224% higher. Interestingly, the relatively inhibitory effect of GF on the mitogenic capacity after short periods of storage can also be seen on the clonogenic capacity. The reason for this inhibitory effect is not fully understood. It may be that the clonogenic and mitogenic effects of GF can be clinically observed after a short period of exposure only at optimal conditions. At non optimal conditions, such as in this study, this effect can be observed only after a certain period of exposure. Another possibility is that after a short period of storage, the effect of GF is expressed mainly by increasing the protein synthesis and not by increasing the mitogenic or clonogenic capacities. However, this function was not evaluated in this study. In addition, the clonogenic capacity represents the ability of stem cells, or very primitive progenitor cells, to proliferate. The nature of these cells is that they are quiescent cells that usually tend to proliferate only after extensive stimulus. It may be that only after a longer period of exposure to GF will the cells start to proliferate. It is difficult to compare this study with others since most of the studies that evaluated the mitogenic effect of GF did so only after 24 h or longer periods of exposure.

Storage media

HBSS is a popular storage medium that has been shown to be effective in preserving avulsed teeth for extended periods (8, 10, 11). In this study, HBSS supplemented with GF was an even more effective medium for preserving the viability, mitogenic and clonogenic capacities of cells especially for a long storage period (24 h) at 24 °C.

Culture medium (Eagle's medium) at 37 °C can preserve PDLF for extended periods (3, 13–15). In this study, α-MEM-S (including all additives: FCS, AB and ribonucleotides) supplemented with GF was the second best medium for preserving the viability, mitogenicity and the clonogenic capacity of PDLF after 24 h of storage at room temperature. This medium was superior to ViaSpan or α MEM.

ViaSpan was shown to decrease the incidence of root resorption after replantation (37) and to be better than milk or HBSS for preserving 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 (10). These results are in agreement with our results in which the viability of PDLF stored for 24 h in HBSS and ViaSpan showed comparable results. However, the values in this study are higher; this might be explained by the different origins of the cells, i.e. explants from very young adults and early passage (3–6, 31). The sum of the functional abilities of PDLF stored in ViaSpan was generally comparable to HBSS or to α-MEM-S. However, the high price (US$300 per liter), the short shelf life (a few months), and the difficult access (only in pharmacies of selected hospitals), make HBSS and α-MEM-S better for storage of avulsed teeth for 2, 8 and 24 h at room temperature.

In conclusion, supplementation of growth factors to storage media improved the effectiveness of the media only when the storage period was long (24 h). For short periods of storage (2 and 8 h), the use of media without GF was preferable. Nevertheless, HBSS followed by α-MEM-S and ViaSpan were the most effective storage media for preserving the viability, mitogenicity and clonogenic capacity of PDLF after

storage for 24 h at 24 °C, whereas α MEM was the least effective medium for preserving these functional abilities. Clonogenic capacity showed a different pattern of results compared to mitogenic capacity. The low reduction in the viability even after 24 h of storage (3%) does not correlate with the low clinical effectiveness of the storage medium.

Before any clinical conclusions are drawn, animal studies must be conducted. There may be a number of reasons for differences between *in vivo* and *in vitro* studies. Firstly, the role of fibroblasts in repopulating denuded root surfaces is not completely clear. Secondly, the study conditions of *in vivo* compared to *in vitro* are different (temperature, air, environment, presence of other biological factors). Moreover, in order to use storage medium supplemented with GF, a kit containing GF has to be manufactured and distributed.

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References

1. Andreasen JO. Etiology and pathogenesis of traumatic dental injuries. A clinical study of 1298 cases. Scand J Dent Res 1970;78:329–42.
2. Andreasen JO, Hjørting-Hansen E. Replantation of teeth. I. Radiographic and clinical study of 110 human teeth replanted after accidental loss. Acta Odontol Scand 1966;24:263–86.
3. Andreasen JO, Reinholdt J, Riis I, Dybdahl R, Soder PO, Otteskog P. Periodontal and pulpal healing of monkey incisors preserved in tissue culture before replantation. Int J Oral Surg 1978;7:104–12.
4. Cvek M, Granath LE, Hollender L. Treatment of non-vital permanent incisors with calcium hydroxide. 3. Variations of occurrence of ankylosis of reimplanted teeth with duration of extra-alveolar period and storage environment. Odontol Revy 1974;25:43–56.
5. Andreasen JO. Effect of extra-alveolar period and storage media upon periodontal and pulpal healing after replantation of mature permanent incisors in monkeys. Int J Oral Surg 1981;10:43–53.
6. Blomlof L. Milk and saliva as possible storage media for traumatically exarticulated teeth prior to replantation. Swed Dent J 1981: 8 (Suppl): 1–26.
7. Ashkenazi M, Marouni M, Sarnat H. *In vitro* viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in four media at room temperature. Endod Dent Traumatol 2000;16:63–70.
8. Hupp JG, Trope M, Mesaros SV, Aukhil I. Tritiated thymidine uptake in periodontal ligament cells of dogs' teeth stored in various media for extended periods. Endod Dent Traumatol 1997;13:223–7.
9. 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: 149–56.
10. Hiltz J, Trope M. Vitality of human lip fibroblasts in milk, Hanks' balanced salt solution and ViaSpan storage media. Endod Dent Traumatol 1991;7:69–72.
11. Trope M, Freidman S. Periodontal healing of replanted dog teeth stored in ViaSpan, milk and Hanks' Balanced Salt solution. Endod Dent Traumatol 1992;8:183–8.
12. Todo S, Nery J, Yanaga K, Podesta I, Gordon RD, Starzl TE. Extended preservation of human liver grafts with UW solution. J Am Med Assoc 1989;261:711–4.
13. Litwin J, Lundquist G, Soder PO. Studies on long-term maintenance of teeth and viable associated cells *in vitro*. Scand J Dent Res 1971;79:536–9.
14. Soder PO, Lundquist G. Autotransplantation of teeth with use of cell cultivation technique. Int Dent J 1972;22:327–40.
15. Nasjleti CE, Castelli, WA, Blankenship JR. The storage of teeth before reimplantation in monkeys. A histologic study. Oral Surg Oral Med Oral Pathol 1975;39:20–9.
16. Terranova VP, Wikesjo UME. Extracellular matrices and polypeptide growth factors as mediators of functions of cells of the periodontium. J Periodontol 1987;58:371–80.
17. Lynch SE. Platelet-derived growth factor and insulin-like growth factor I. Mediators of healing in soft tissue and bone wounds. Periodont Case Rep 1991;13:13–20.
18. Lynch SE, Williams RC, Polson AM, Howell TH, Reddy MS, Zappa UE, et al. A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. J Clin Periodontol 1989;16:545–8.
19. Lynch SE, de Castilla GR, Williams RC, Kiritsy CP, Howell TH, Reddy MS, et al. The effect of short term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. J Periodontol 1991;62:458–67.
20. Matsuda N, Lin W-L, Kumar NM, Cho MI, Genco RJ. Mitogenic, chemotactic and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors *in vitro*. J Periodontol 1992;63:515–25.
21. Oates TW, Rouse CA, Cochran DL. Mitogenic effects of growth factors on human periodontal ligament cells *in vitro*. J Periodontol 1993;64:142–8.
22. The American Academy of Periodontology. A possible role for poly-peptide growth factors and differentiation factors in periodontal regeneration (position paper). Chigaco: The American Academy of Periodontol; April, 1990.
23. Morris ML, Moreinis AR, Prestup A. Factors affecting healing after experimentally delayed tooth transplantation. J Endod 1981;7:80–4.
24. Lekic P, Kenny D, Moe HK, Barrett E, McCulloch CAG. Relationship of clonogenic capacity to plating efficiency and vital dye staining of human periodontal ligament cells: implication for tooth replantation. J Periodontal Res 1996;31:294–300.
25. Philips HJ. Dye exclusion tests for cell viability. In: Kruse PF, Patterson MK, editors Tissue culture methods and applications. New York: Academic Press; 1973. p. 406–8.
26. Mariotti A, Cochran DL. Characterization of fibroblasts derived from human periodontal ligament and gingiva. J Periodontol 1990;61:103–11.
27. Lekic PC, Kenny DJ, Barrett EJ. The influence of storage conditions on the clonogenic capacity of periodontal ligament cells: implications for tooth replantation. Int Endod J 1998; 31:137–40.
28. Andreasen JO, Borum MK, Jakobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 4. Factors related to periodontal ligament healing. Endod Dent Traumatol 1995;11:76–89.
29. Weinstein FM, Worsaae N, Andreasen JO. The effect on periodontal and pulpal tissues of various cleansing procedures prior to replantation of extracted teeth. An experimental study in monkeys. Acta Odontol Scand 1981;39:251–5.
30. Matsson L, Andreasen J, Cvek M, Granath L. Ankylosis of experimentally reimplanted teeth related to extra-alveolar period and storage environmental. Pediatr Dent 1982;4:327–9.
31. Sodek J. A comparison of the rates of synthesis and turnover

of collagen and non-collagen proteins in adult rat periodontal tissues and skin using a microassay. Arch Oral Biol 1977; 22:655–65.
32. Matsuda N, Lin WL, Kumar NM, Cho MI, Genco RJ. Mitogenic, chemotactic, and synthetic response of rat periodontal ligament fibroblast cells to polypeptide growth factors *in vitro*. J Periodotol 1992;63:515–25.
33. Rutherford RB, Niekrash CE, Kennedy JE, Charette MF. Platelet-derived and insulin–like growth factors stimulate regeneration of periodontal attachment in monkeys. J Periodont Res 1992;27:285–90.
34. Wang HL, Pappert TD, Castelli WA, Chiego DJ Jr., Shyr Yu, Smith BA. The effect of platelet-derived growth factor on the cellular response of the periodontium: an autoradiographic study on dogs. J Periodontol 1994;65:429–36.
35. Blom S, Holmstrup P, Dabelsteen E. A comparison of the effect of epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor on rat periodontal ligament fibroblasts-like cells' DNA synthesis and Morphology. J Periodontol 1994;65:373–8.
36. Oates TW, Rouse CA, Cochran DL. Mitogenic effect of growth factors on human periodontal ligament cells *in vitro*. J Periodontol 1993;64:142–8.
37. Andreasen JO, Borum MK, Jakobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 4. Factors related to periodontal ligament healing. Endod Dent Traumatol 1995;11:76–89.