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Effect of temperature and storage media on human periodontal ligament fibroblast viability

Beatriz Dulcineia Mendes Souza[1], Débora Denardin Lückemeyer[2], Wilson Tadeu Felippe[1], Cláudia Maria Oliveira Simões[2], Mara Cristina Santos Felippe[1]

Departments of [1]Dentistry and [2]Pharmaceutical Sciences, Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil

Correspondence to: Mara Cristina Santos Felippe, Presidente Coutinho, 179/702 Centro, Florianópolis, CEP: 88.015-230, SC, Brazil
Tel./Fax: +55 (0) 48 33240147
e-mail: mcsfelippe@hotmail.com

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Abstract – Many solutions have been examined as possible storage media for avulsed teeth. The purpose of this study was to compare the effectiveness of several storage media to preserve cultured periodontal ligament fibroblasts (PDLF) under different temperatures. The media tested were: sterile Hank's balanced salt solution (sHBSS), non-sterile HBSS (nHBSS), skimmed milk, Save-A-Tooth®, Minimum Essential Medium (MEM) and water (negative control). MEM at 37°C was used as positive control. PDLF were obtained from explants of extracted healthy human teeth. Plates containing confluent PDLF were soaked in the various media for 3, 6, 24, 48 and 72 h at 37°C and 20°C. After incubation, viability of the cells was determined using the tetrazolium salt-based colorimetric (MTT) assay and the Trypan Blue exclusion test after 6, 24, 48 and 72 h of incubation at 20°C. The results were analyzed statistically using Kruskal–Wallis, Scheffé and Mann–Whitney ($\alpha = 5\%$) tests. Results from the MTT assay at 37°C and 20°C showed that skimmed milk was the best storage medium for up to 24 and 48 h, respectively, followed by nHBSS and sHBSS. Results from the Trypan Blue exclusion test showed that the best storage media were milk, sHBSS and nHBSS, with no statistical differences, for any time period. The Save-A-Tooth® had a detrimental effect on cells after 24 h. The influence of temperature on the effectiveness of the storage media tested showed at 20°C a decreasing order of efficacy as follows: milk > sHBSS and nHBSS > MEM > Save-A-Tooth® > water while at 37°C it was: MEM > nHBSS > milk > sHBSS > Save-A-Tooth® > water. In conclusion, incubation temperature altered the effectiveness of the storage media and skimmed milk at 20°C was better than HBSS in maintaining PDLF viability.

Avulsions occur in 0.5–3% of all traumatic injuries to the permanent dentition (1). Studies have shown that a dry period of 2 h results in necrosis of almost all of the periodontal ligament cells (2, 3). It is therefore recommended that the tooth is re-implanted as quickly as possible (4). When this is not possible, the tooth must be stored in an adequate medium to preserve it (5). Several experiments have been carried out in an attempt to find the ideal medium (4–6). Possible candidates include: Eagle's medium, Viaspan®, water, milk and Hank's balanced salt solution (HBSS). Although Eagle's medium and Viaspan® have the capacity to maintain cell viability for a longer period (4, 6, 7), they are expensive products that are not readily available. Tap water is an inadequate medium because it has low osmolality and contains chlorine (2, 8–10).

HBSS is the storage medium of choice for avulsed teeth (11) and is commercially available as Save-A-Tooth® (Phoenix-Lazerus, Shartlesville, PA, USA). Although several studies have shown good results with HBSS (4–6, 10, 12, 13), its widespread use has not been realized because it is not widely available in pharmacies or drug stores (14).

Milk has gained wide acceptance as a storage medium as a result of its low bacterial content, and because it provides some nutrients (5) and growth factors to the cells (15). However, the time-period over which cell viability is maintained in milk is controversial. Some studies show that it is effective for 3 (12, 16) or 6 h (4–6), others show effectiveness for up to 24 h (13). There is also no consensus when the effectiveness of milk is compared with other media, in particular HBSS (6, 9, 12, 14). While Hiltz and Trope (6), and Huang et al. (12) verified that milk was not as effective as HBSS, Olson et al. (14) and Marino et al. (9) obtained better results with milk.

After establishing a strain of periodontal ligament fibroblasts (PDLF), the objectives of this study were to compare the effectiveness of different storage media in maintaining cell viability, and to verify the effect of different temperatures (20°C and 37°C) on the effectiveness of the media studied.

Materials and methods

Cell culture

The procedure for the primary culture and establishment of cells was carried out according to Sant'Ana et al. (17), with modifications. The PDLF were obtained from

clinically healthy third molar teeth extracted for orthodontic reasons. The periodontal ligament of the middle third of the roots was scraped and placed in culture flasks containing MEM (Cultilab, Campinas, SP, Brazil), 10% fetal bovine serum (FBS) (Cultilab) and 1% penicillin G sodium (10 000 IU), streptomycin (10 mg) and amphotericin B (25 μg) (collectively, PSA) (Cultilab). The cultures were incubated at 37°C with 5% CO_2 in a humidified atmosphere. Once confluent, the explant was removed, the cells were trypsinized (Trypsin, Sigma Chemical CO., St Louis, MO, USA) and subcultivated (first passage). The medium was renewed every 3 days. To guarantee the establishment of this PDLF strain, aliquots of cellular suspensions were frozen in liquid nitrogen (MVE Inc., New Prague, MN, USA) at −126°C. Cells from passages 5–10 were used in the experiments. The tetrazolium salt-based colorimetric (MTT) assay and the Trypan Blue dye exclusion method were used for cell viability evaluation, as described below.

MTT assay

PDLF (8×10^3 cells per well) were seeded in ten 96-well culture plates (TPP, Trasadingen, Switzerland) and incubated at 37°C with 5% CO_2. At confluence, MEM was removed, and the wells were filled with 100 μl of one of the following solutions: MEM filtered through a 0.22 μm filter (Millipore, Chicago, IL, USA) and supplemented with 2% FBS and 1% PSA (pH 7.3); tap water (pH 7.6); Save-A-Tooth® (Phoenix-Lazerus) (pH 6.4); sterile HBSS (sHBSS) sterilized using a 0.22 μm filter (pH 7.0); non-sterile HBSS (nHBSS) (pH 7.0); and skimmed pasteurized long-life milk (Parmalat, São Paulo, SP, Brazil) (pH 6.8).

HBSS was prepared according to the Save-A-Tooth® manufacturer's formula: sodium chloride (8 g/l), D-glucose (0.4 g/l), potassium chloride (0.4 g/l), sodium bicarbonate (0.35 g/l), sodium phosphate (0.09 g/l), potassium phosphate (0.14 g/l), calcium chloride (0.14 g/l) and magnesium sulfate (0.1 g/l). The concentrations were based on formula presented by Krasner and Person (18).

Five of the 10 plates were incubated at 37°C and five were kept at 20°C. After 3, 6, 24, 48 and 72 h the storage media were replaced by MTT solution (1 mg/ml) (Sigma), and the plates were incubated at 37°C. After 4 h, the MTT solution was removed, and 100 μl of dimethylsulfoxide (DMSO) was added to the wells. Cell viability was determined by measuring the optical density at 540 nm on a spectrophotometer (Bio-Tek Instruments-Inc., EL_x 800, Winooski, VT, USA). The absorbance values, after cells had been stored in MEM at 37°C (MEM-37), were used as positive control for cell growth.

Trypan Blue dye exclusion method

This method was carried out as proposed by Walum et al. (19), with minor modifications. PDLF (8×10^4 cells per well) were seeded into 24-well culture plates (TPP), which were incubated at 37°C in an atmosphere of 5% CO_2. After 24 h, cells were washed with PBS and four wells in each column were filled with 1 ml of MEM, Save-A-Tooth®, sHBSS, nHBSS or skimmed milk.

The plates were kept at 20°C for 6, 24, 48 and 72 h, after which the cells were collected and labeled with 0.4% of Trypan Blue solution (Sigma). Enumeration of viable and non-viable cells was carried out using a light microscope with a hemocytometer at 40 × magnification (TNM, Nikon, Tokyo, Japan).

Statistical analysis

Statistical analysis of the data was accomplished using the Kruskal–Wallis test, complemented by the Scheffé test. The Mann–Whitney test was used to analyze the results obtained from the MTT assay at 20°C and 37°C. The level of significance was 5%.

Results

At first, it was established a strain of PDLF. The primary culture was obtained in 10 days and the subcultures, which allowed the establishment of the cell strain, were obtained after an average period of 15 days. These cells were then used in assays to compare the effectiveness of different storage media, and different storage temperatures, on maintaining cell viability.

Cell viability determined by MTT assay

The mean absorbance values, which represent PDLF viability for each tested media and for each storage period, are shown in Fig. 1 (37°C) and Fig. 2 (20°C). The Kruskal–Wallis test shows time dependent results according to the experimental solution analyzed ($P < 0.001$).

In the experiments carried out at 37°C, the efficacy of milk at 3, 6 and 24 h was significantly higher than sHBSS and nHBSS ($P < 0.001$). When HBSS solutions were compared, there was no difference at 3, 6 and 24 h, whereas, at 48 and 72 h, nHBSS was significantly more effective than sHBSS at maintaining cell viability. Save-A-Tooth® was significantly less effective than milk, sHBSS, nHBSS and MEM-37 ($P < 0.001$) at every time period.

At 20°C, milk was significantly more effective than sHBSS and nHBSS ($P < 0.001$) for up to 48 h. At 72 h, there was no significant difference between these media. When HBSS solutions were compared, there was no difference between them. The efficacy of Save-A-Tooth® and MEM at 3 and 6 h was similar to sHBSS and nHBSS, but at 24, 48 and 72 h these media were significantly less effective at maintaining cell viability than milk, sHBSS, nHBSS and MEM-37 ($P < 0.001$).

Comparison of results obtained with MTT assays at 37°C and 20°C

The storage of PDLF at different temperatures revealed significantly different absorbance values depending on the medium and the period evaluated. Generally, MEM and nHBSS media incubated at 37°C were significantly more effective at maintaining cell viability than these same media at 20°C ($P < 0.001$). Similarly, milk and sHBSS media were more effective at 37°C than at 20°C

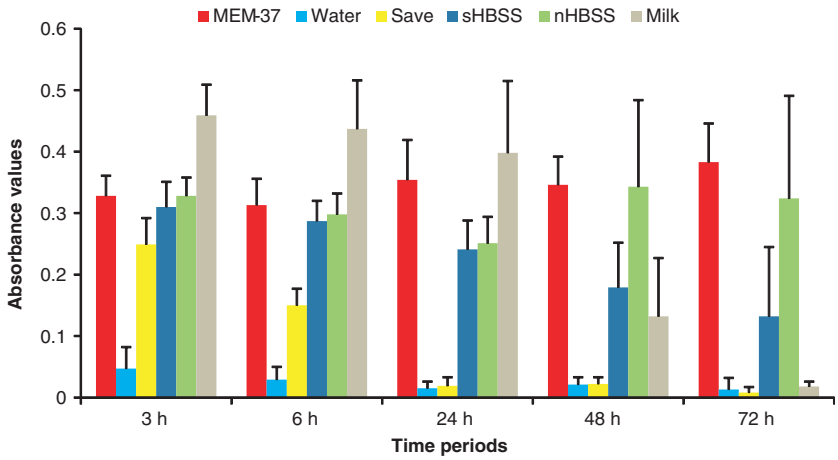

Fig. 1. Results for the viability of periodontal fibroblasts conserved at 37°C, obtained by the colorimetric MTT method, expressed as absorbance values.

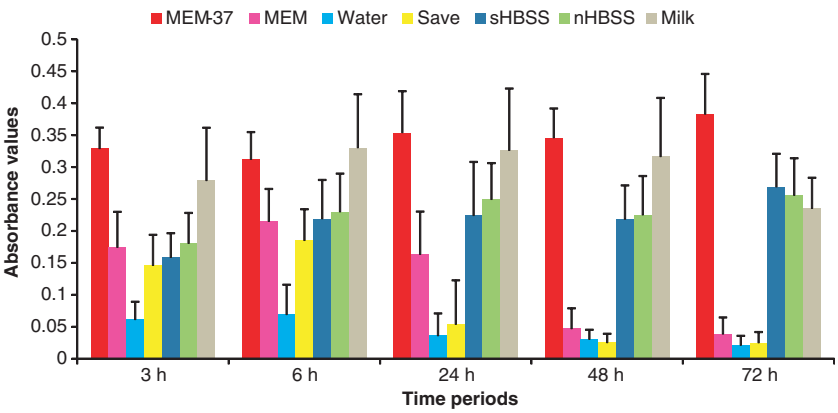

Fig. 2. Results for the viability of periodontal fibroblasts conserved at 20°C, obtained by the colorimetric MTT method, expressed as absorbance values.

for up to 24 h. Water and Save-A-Tooth® were less effective at maintaining cell viability at 37°C than at 20°C.

Cell viability determined by the Trypan Blue dye method

The percentage averages (±SD) of cell viability for different media and different periods of storage are shown in Fig. 3. The efficacy of Save-A-Tooth® and MEM at 24, 48 and 72 h was significantly lesser than that of sHBSS, nHBSS and milk. At 6 h, there was no significant difference between these media. There were no significant differences in efficacy between milk, sHBSS and nHBSS.

Discussion

The aim of this study was to compare the effectiveness of different storage media in maintaining the viability and mitochondrial functioning of PDLF.

Studies have demonstrated that storage time can influence the effectiveness of different media (6, 12–14, 16). In this study, it was used time-periods from 3 to 72 h, because these are considered clinically relevant.

Although body temperature is 37°C, an avulsed tooth is normally stored in a medium at room temperature. As there are experiments carried out at 37°C (5, 8, 9, 16, 20) and at room temperature (6, 21), this study was performed under both conditions to allow comparisons.

Ashkenazi et al. (21) demonstrated that cells cultivated in a culture medium at 37°C have greater mitogenic and clonogenic capacity than those cultivated at room temperature. Based on this finding, and according to the methodology employed by other authors (5, 13, 21), MEM at 37°C was used as positive control in the MTT assay carried out at both temperatures. Although MEM-37 maintained a greater percentage of metabolically active cells for 24, 48 and 72 h, its performance was statistically poorer than that of milk in the initial periods (3 and 6 h). In this experiment, FBS was added to MEM at a concentration of 2%. Although this concentration was shown to be sufficient for the maintenance of cell viability at 37°C, it is possible that the results would have been improved if a greater concentration had been used (5, 9, 10, 13, 21). Tap water was used as negative control at 37°C and 20°C, and showed the lowest efficacy, in agreement with previous studies (2, 8–10).

The results of the experiment carried out at 20°C revealed that, up to 48h, milk was significantly more efficient at maintaining cell viability than other tested media, and after 72 h its results were equivalent to those of sHBSS and nHBSS. Although these results are partially in agreement with the findings of Olson et al.

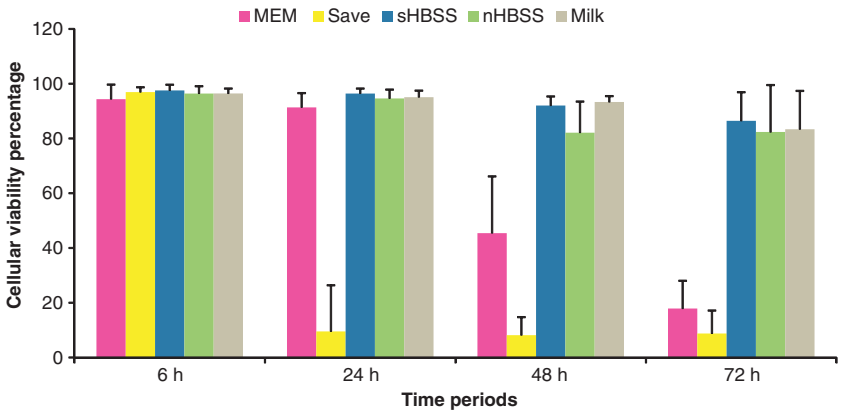

Fig. 3. Results for the viability of periodontal fibroblasts conserved at 20°C, obtained by the Trypan Blue dye exclusion method, expressed in percentages.

(14) and Marino et al. (9), they differ from those of other authors, who stated that milk is effective for a short period of time (12, 16), and who affirmed that only cold milk (4°C) is suitable for the preservation of the proliferation capacity of periodontal ligament cells (10, 13). When the experiments were performed at 37°C, milk was even more efficient at maintaining cell viability up to 24 h. One possible explanation for the positive results obtained with milk at both tested temperatures is that, besides the physiological pH and osmolarity and the presence of some nutrients (5), it contains growth factors (15). Belford et al. (15) demonstrated that the addition of bovine milk extracts to cultures of fibroblasts and epithelial cells provided greater cell proliferation. It may also be suggested that the relative opacity of milk interfered in optical density reading. Smee et al. (22) stated that colored substances can interfere in the absorbance readings generating higher values. On testing different concentrations of chlorophyllin added to HBSS, Chung et al. (20) raised the hypothesis that the green color of this substance may have increased the absorbance values overestimating the results. However, the results of this study with the Trypan Blue exclusion method confirmed the effectiveness of milk in the maintenance of cell viability. Interestingly, the effectiveness of milk at 37°C decreased considerably from 48 h onwards being equal to that of water after 72 h. Probably, after 48 h, milk suffered a reduction in pH, generating an inappropriate environment for cell survival.

HBSS was tested in the sterile and non-sterile forms to evaluate whether a prior sterilization would affect its function. The sterilization of HBSS cannot be carried out in an autoclave because of the presence of glucose. Therefore, 0.22 μm filters adapted in specific devices were used, which made preparation of the solution more laborious, time-consuming and expensive. The results of this study show that there are no advantages of sterilizing the product, because the performance of both solutions was similar for all time-periods, particularly at 20°C.

In this study, cell culture at 20°C and 37°C showed discouraging results with Save-A-Tooth®. After the initial periods (3 and 6 h) of cultivation at 20°C, Save-A-Tooth® maintained around 50% cell viability. At 37°C, the percentage of viable cells in relation to the positive control was approximately 75% (3 h) and 48% (6 h) (data not shown). After 24, 48 and 72 h, at both temperatures, the results were statistically the same as those for water. Other studies carried out with this product revealed similar results (9, 14).

After cell storage in whole milk and in Save-A-Tooth®, Olson et al. (14) and Marino et al. (9) verified that whole milk showed a significantly better performance than Save-A-Tooth® and the positive control, after 4 and 8 h, respectively. Olson et al. (14) verified that after 8 and 12 h, the average values for optical density of cells maintained in Save-A-Tooth® were similar to those shown by cells maintained in a dry environment. In this study, Save-A-Tooth® was stored at room temperature and used approximately 6 months after its acquisition. It is possible that storage of this product might have influenced the results obtained, because HBSS used immediately after its preparation gave a better performance than Save-A-Tooth®. It must be emphasized that although these products had the same components, the concentration of each one is not specified by Save-A-Tooth®'s manufacturer. A study is being carried out using HBSS after storage for 6 months and 1 year to address this issue. It should be noted that the pH of sHBSS and nHBSS was 7.0, while it was 6.4 for Save-A-Tooth®, which is not ideal for cell growth.

The results obtained with Trypan Blue test were similar with those of the MTT assay. Milk was the best storage medium, and Save-A-Tooth® and MEM showed a significant reduction in their effectiveness after 24 and 48 h, respectively. However, sHBSS and nHBSS showed similar performance than milk, suggesting that the Trypan Blue dye method is less sensitive than the MTT assay. Ashkenazi et al. (13, 21) tested cell viability using the Trypan Blue dye method and verified that there were no significant differences between the tested media after 2, 8 and 24 h. However, on testing cell mitogenic and clonogenic capacity, the authors found significant differences after 24 h. The sensitivity of this method could be low because it does not characterize the metabolic condition of the non-stained cells, which means that the plasma membrane may remain intact, without the cell having any metabolic activity (23, 24).

The results of this study differ from those of Hiltz and Trope (6) who also used the Trypan Blue dye exclusion

method, and verified that cell storage at room temperature in milk and in HBSS was effective for 6 and 24 h, respectively. This disparity may be due to the origin of the fibroblasts and the type of milk used. Those authors carried out the study on labial fibroblasts and whole milk, whereas this study used periodontal fibroblasts and skimmed milk, which is recommended since it has less fat content (8).

In conclusion, temperature affected the efficacy of storage media at maintaining PDLF viability. The effectiveness of the storage media tested at 20°C in decreasing order was as follows: milk > sHBSS and nHBSS > MEM > Save-A-Tooth® > water. Considering that normally avulsed teeth are maintained in a medium at room temperature, and in view of the findings from this study, it seems that skimmed milk at 20 °C is a good storage medium for up to 48 h. Perhaps, replacement of the milk at regular intervals of 24 or 48 h could increase its effectiveness further. A study is being conducted to evaluate this.

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