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Effect of milk renewal on human periodontal ligament fibroblast viability *in vitro*

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Abstract – Milk has been studied extensively and has gained wide acceptance as a suitable storage medium capable of maintenance of avulsed teeth that cannot be replanted immediately. The objective of this study was to evaluate whether the renewal of milk as a storage medium every 24 h for up to 120 h is able to increase its ability to maintain human periodontal ligament fibroblasts (PDLF) viability *in vitro*. Plates with confluent PDLF were soaked in minimum essential medium (MEM) at 37°C (positive control) and in skimmed milk (22 wells) and water (negative control) for 24, 48, 72, 96, and 120 h at 5 and 20°C. The skimmed milk was renewed every 24 h in 11 of the wells of each plate. After these periods, cell viability was determined by the tetrazolium salt-based colorimetric (MTT) assay. Data were statistically analyzed by Kruskal–Wallis and Scheffé tests ($\alpha = 5\%$). At 24 h, milk and MEM performed similarly. However, from 48 h onwards, MEM was significantly better than renewed and not renewed milk at both temperatures. Regardless of temperature (5 or 20°C), renewal of milk with fresh milk did not affect its ability to maintain PDLF viability.

When dental avulsion occurs, the recommended practice is to replant the tooth immediately or as quickly as possible to avoid adverse occurrences, including root resorption (1, 2). When this is not practical, storing the avulsed tooth in an adequate medium (3) can maintain the viability of the periodontal ligament cells, allowing a more favorable prognosis when the tooth is eventually replanted.

Milk has been studied extensively (3–15) in this regard and is widely accepted as a suitable transport medium for avulsed teeth (16). It has low bacterial content (3, 5), physiologic osmolality (230–270 mOsm Kg^{-1}), and neutral pH (6.5–6.8) and provides some nutrients (3) and growth factors to the cells (11, 12). In relation to the time for which cell viability is maintained, *in vitro* studies with cultured cells have shown controversial results (4–6, 10, 13). While some studies demonstrated effectiveness for only up to 3 h (4, 6) or 6 h (5), others showed effectiveness for 24 h (13) or for up to 48 h (10). There is also no consensus regarding the effectiveness of milk as storage medium when compared to other media, mainly with Hank's balanced salt solution (HBSS) (6, 8, 10, 14). While Huang et al. (6) verified that milk was not as effective as HBSS, other authors obtained better results with milk (8, 10, 14). Souza et al. (10) verified that when culture plates were kept at 20 and 37°C, milk was the best storage medium for up to 48 and 24 h, respectively. The authors supposed that after these periods, the pH of the milk decreased, creating an unsuitable environment for cell survival (3).

To date, no research has been conducted to determine whether regular renewal of the milk impacts on its ability to maintain the viability of human periodontal ligament fibroblasts (PDLF). Thus, the objective of this study was to evaluate whether daily renewal of the milk has influence on the viability of PDLF *in vitro*.

Materials and methods

The project was approved by the Ethics Committee for Research with Human Beings of the Federal University of Santa Catarina (UFSC) (Protocol 074/08).

PDLF harvested from clinically healthy third molar teeth were cultured in culture flasks with minimum essential medium (MEM) (Cultilab, Campinas, SP, Brazil) containing 10% fetal bovine serum (FBS) (Cultilab) and 1% of penicillin G sodium (10 000 UI), streptomycin (10 mg), and amphotericin B (25 μg) (collectively, PSA) (Cultilab) in a humidified incubator with 5% CO_2 and 95% air at 37°C (Fanem, HF 212, São Paulo, SP, Brazil). The cells were subcultured every 4 days until an adequate number of cells were obtained. Cells from passages five to ten were used in the experiments.

For the experiment, the cells (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 from 33 of the wells, in each plate. Twenty-two of these were then filled with 100 μl of skimmed pasteurized long-life milk (Parmalat, São Paulo, SP, Brazil) (pH 6.8) from a newly opened package maintained at room temperature. The remaining 11 wells were filled with 100 μl of tap water (pH 7.6) as a negative control. Five of the ten plates were kept at 5°C and the other five at 20°C. Every 24 h, the milk in 11 wells of each plate was carefully aspirated and immedi-

Table 1. Absorbance and standard deviation (±SD) values of all media and time periods*

Storage media	24 h	48 h	72 h	96 h	120 h
MEM-control	0.33 ± 0.11 (a)**	0.40 ± 0.08(a)	0.54 ± 0.03(a)	0.50 ± 0.06(a)	0.52 ± 0.04(a)
Milk −20°C	0.34 ± 0.07(a)	0.34 ± 0.06(b)	0.33 ± 0.05(b)	0.31 ± 0.09(b)	0.18 ± 0.09(c)
R Milk −20°C	–	0.24 ± 0.04(c)	0.28 ± 0.06(c)	0.27 ± 0.08(c)	0.10 ± 0.03(d)
Milk −5°C	0.33 ± 0.05(a)	0.32 ± 0.03(b)	0.32 ± 0.08(b)	0.32 ± 0.03(b)	0.25 ± 0.06(b)
R Milk −5°C	–	0.32 ± 0.03(b)	0.30 ± 0.06(c)	0.28 ± 0.02(c)	0.22 ± 0.03(b)
Water	0.04 ± 0.02(b)	0.03 ± 0.02(d)	0.02 ± 0.01(d)	0.04 ± 0.02(d)	0.03 ± 0.02(e)

*Initial absorbance value = 0.36.
**Different letters indicate statistically significant difference ($P < 0.05$) at the same periods of time.

ately replaced with 100 ml of milk from newly opened packages, while the milk in the remaining 11 wells of each plate was left *in situ*. Five plates with confluent PDLF were soaked in MEM at 37°C as positive control for cell growth.

After 24, 48, 72, 96, and 120 h, the storage media in two of the plates (one left at 5°C and another at 20°C) were replaced by MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical CO., St. Louis, MO, USA), and the plates were incubated at 37°C. After 4 h, the MTT solution was removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to the wells. Cell viability was determined by measuring the optical density at 540 nm on a spectrophotometer (EL_x 800; Bio-Tek Instruments-Inc., Winooski, VT, USA). Statistical analysis of the data was accomplished using the Kruskal–Wallis test, complemented by the Scheffé test. The level of significance was 5%.

Results

The initial absorbance value was 0.36. The mean absorbance values, which represent PDLF viability for each media tested at different temperatures and storage periods, are shown in Table 1 and Fig. 1. The Kruskal–Wallis test showed time-dependent results according to the experimental solution analyzed ($P < 0.001$).

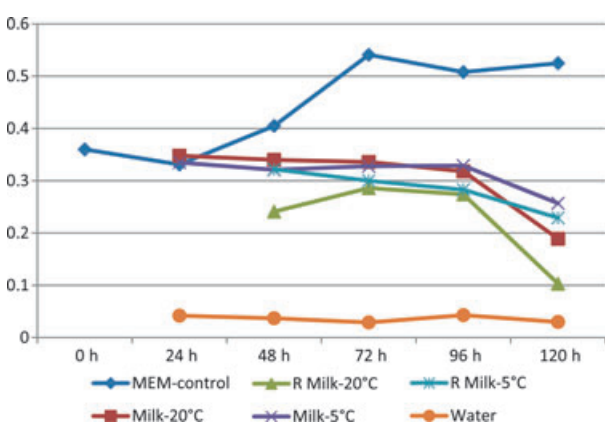

Fig. 1. Viability, expressed as absorbance values, of PDLF conserved at 5 and 20°C using different media and time periods. (RMilk: replaced milk).

The results indicated that MEM, renewed milk, and not renewed milk performed significantly better than the negative control at all time periods and temperatures ($P < 0.001$). At 24 h, MEM effectiveness was similar to that of cold (5°C) and room-temperature milk (20°C). However, from 48 h onwards, viability of the cells stored in MEM was significantly higher than that stored in renewed and not renewed milk ($P < 0.001$) at both temperatures. Regardless of temperature (5 or 20°C), milk renewal was not able to increase PDLF viability.

Discussion

In addition to preserving cell viability, milk is relatively cheap and can be easily found in places where avulsion commonly occurs. Skimmed milk was used in this study because Harkacz et al. (7) showed that milk with a lower fat content might be more appropriate in maintaining PDL cell viability than milk with a higher fat content. Using different storage media at 37 and 20°C, Souza et al. (10) showed that skimmed milk was significantly better than all other tested solutions for up to 24 and 48 h, respectively. Considering that the decline in the performance of milk over time may have occurred because of its pH drop (3, 10), it was suggested that its renewal every 24 h could increase its effectiveness (10). Thus, the objective of this study was to evaluate whether daily milk renewal has influence on PDLF viability *in vitro*, by the analysis of cell mitochondrial function using MTT assay. The results of the experiment carried out at 5 and 20°C revealed that milk renewal promotes a negative effect on cell viability. It could be hypothesized that the removal of growth factors and chemical cell mediators (proteins), by replacing the milk, may have disturbed cellular metabolism and the reversion of tetrazolium salts in formazan crystals. It is known that the cells interact with each other and with the extra cellular components promoting important activities such as cell migration, growth, and differentiation. These interactions may occur through the actions of proteins secreted by the cells, or may be the result of the contact between them (17). It is also possible that, at the time of replacement, the act of aspiration may have had a negative effect on the confluent monolayer of cells.

It must be emphasized that as this research is based on *in vitro* cell culture and that the conditions of PDLF may not be comparable with the conditions of the cells adhered to the root surface of a real avulsed tooth. So, considering the amount of collagen surrounding the cells,

it is possible that in a clinical situation, milk renewal does not affect the cells as much as in this *in vitro* situation.

Avulsed teeth are often not replanted at the site of the accident (18). In some cases, patients suffer more serious injuries than dental avulsion, remaining in hospital unable to receive an appropriate dental treatment. In these situations, the avulsed teeth should be placed in a physiologic environment for a few days until replantation. Thus, this study was carried out considering storage periods from 24 to 120h to be clinically relevant.

Generally, avulsed teeth are stored in a medium at room temperature. However, it is widely acknowledged that low temperatures have the advantage of reducing cellular metabolism (19), limiting bacterial growth, and preventing milk from 'souring', which may be significant after replantation (13). Previous studies have shown that storage of PDL cells in cold milk is advantageous (3, 9, 13). Thus, in this study, the daily renewal of the milk was carried out at 5 and 20°C to allow comparisons. The results showed that cells maintained in not renewed milk at both temperatures demonstrated similar viability (Table 1). However, at 120 h, not renewed milk showed better results when kept at 5°C. It seems that this finding is not relevant as at this time period, the performance of not renewed milk was 50% lower than that of MEM. Concerning renewed milk, the replacement of milk kept at 5°C was less detrimental to cells than the replacement of milk kept at 20°C, especially at 48 h and 120 h. The lower temperature probably provided greater stability of the confluent cells, preventing their detachment.

The results of this study were quite different from Souza et al. (10), who showed that milk, at 20°C, was a suitable media for up to 48 h. In this study, in both temperatures the performance of not renewed milk was similar from 24 to 96 h. The reason for the differing results is unknown.

In conclusion, the effectiveness of the storage media tested in decreasing order was as follows: MEM > not renewed milk > renewed milk > tap water. Perhaps, renewal of the milk at greater intervals may compensate its pH drop and may cause lesser damage on the organization of the confluent monolayer cells. A study is being carried out to address this issue.

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