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Viability of human periodontal ligament fibroblasts in milk, Hank's balanced salt solution and coconut water as storage media

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Abstract

Souza BDM, Lückemeyer DD, Reyes-Carmona JF, Felippe WT, Simões CMO, Felippe MCS. Viability of human periodontal ligament fibroblasts in milk, Hank's balanced salt solution and coconut water as storage media. *International Endodontic Journal*, **44**, 111–115, 2011.

Aim To evaluate the effectiveness of various storage media at 5 °C for maintaining the viability of human periodontal ligament fibroblasts (PDLF).

Methodology Plates with PDLF were soaked in recently prepared Hank's balanced salt solution (HBSS), skimmed milk, whole milk, Save-A-Tooth® system's HBSS (Save), natural coconut water, industrialized coconut water or tap water (negative control) at 5 °C for 3, 6, 24, 48, 72, 96 and 120 h. Minimum essential medium (MEM) at 37 °C served as the positive control. PDL cell viability was determined by MTT assay. Data were statistically analysed by Kruskal–Wallis test complemented by the Scheffé test ($\alpha = 5\%$).

Results The greatest number of viable cells was observed for MEM. Skimmed and whole milk, followed by natural coconut water and HBSS, were the most effective media in maintaining cell viability ($P < 0.05$). From 24 to 120 h, Save, industrialized coconut water and tap water were the worst storage media.

Conclusions Skimmed and whole milk had the greatest capacity to maintain PDLF viability when compared with natural coconut water, HBSS, Save, industrialized coconut water and tap water.

Keywords: avulsion, coconut water, periodontal ligament fibroblasts, storage media.

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Introduction

Tooth avulsion is characterized by complete displacement of a tooth from its alveolar socket. During the extra-alveolar period, adherent cells on to the root are subject to contamination and dehydration and might become necrotic (Andreasen 1981, Doyle *et al.* 1998). Thus, it is recommended to replant the tooth as quickly as possible (Blomlöf *et al.* 1983, Trope & Friedman 1992). However, immediate repositioning of teeth is not always possible, so the choice of a suitable storage medium for maintenance of PDL cell viability is of extreme importance for the success of replantation.

Hank's balanced salt solution (HBSS), commercially available in the Save-A-Tooth® system (Phoenix-Lazerus, Shartlesville, PA, USA), has been recommended as the storage medium to maintain PDL cell viability (American Association of Endodontists 1995). Several experiments have shown that it is an effective medium for the storage of avulsed teeth (Blomlöf 1981, Hiltz & Trope 1991, Krasner & Person 1992, Trope & Friedman 1992, Huang *et al.* 1996, Ashkenazi *et al.* 2000), for periods varying from 3 to 72 h (Hiltz & Trope 1991, Huang *et al.* 1996, Ashkenazi *et al.* 1999, 2000). A disadvantage of HBSS is that it may not be readily available in many locations in which tooth avulsions are likely to occur.

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Milk has been studied extensively and has gained wide acceptance as a storage medium (Blomlöf & Otteskog 1980, Blomlöf 1981, Lindskog *et al.* 1983, Huang *et al.* 1996, Harkacz *et al.* 1997, Marino *et al.* 2000, Sigalas *et al.* 2004, Souza *et al.* 2010a). It has been suggested that its effectiveness can last from 3 to 48 h (Blomlöf & Otteskog 1980, Blomlöf *et al.* 1983, Huang *et al.* 1996, Ashkenazi *et al.* 1999, Souza *et al.* 2010a) and that milk with a lower fat content might be more appropriate in maintaining PDL cell viability than milk with a higher fat content (Harkacz *et al.* 1997).

As it has almost the same isotonicity as sports drinks, coconut water has been used for rehydration and salt replacement. This natural isotonic fluid is available in its natural form directly from the coconut or in long-shelf-life packages (industrialized form) mainly in tropical countries (Gopikrishna *et al.* 2008a,b). Coconut water has recently been studied as a storage medium but with controversial results (Gopikrishna *et al.* 2008a,b, Moreira-Neto *et al.* 2009). Whilst Gopikrishna *et al.* (2008a,b) reported that coconut water was superior to HBSS and milk in maintaining human fibroblast cell viability, Moreira-Neto *et al.* (2009) found that this product was worse than milk. It must be emphasized that, besides other methodological differences, Gopikrishna *et al.* (2008a,b) used natural coconut water at room temperature and Moreira-Neto *et al.* (2009) used long-shelf-life (industrialized) coconut water at 37 °C.

Several studies have shown that the temperature of the storage medium affects the viability of PDL cells (Blomlöf & Otteskog 1980, Blomlöf 1981, Lekic *et al.* 1998, Schwartz *et al.* 2002, Sigalas *et al.* 2004). Sigalas *et al.* (2004) showed that a lower temperature has a positive effect on cellular viability maintenance. Previous studies indicate that cold milk is more effective than milk at room temperature (Blomlöf & Otteskog 1980, Blomlöf 1981, Ashkenazi *et al.* 1999, Sigalas *et al.* 2004).

The purpose of this study was to compare, at 5 °C, the effectiveness of skimmed milk, whole milk, Save-A-Tooth® system's HBSS, recently prepared HBSS, natural coconut water and industrialized coconut water in maintaining PDLF viability over time, by the analysis of cell metabolism using MTT assay.

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 073/08). Procedures for the primary culture and establishment of the cell strain were carried out according to a technique described by Sant'ana *et al.* (2002) and modified by Souza *et al.* (2010a).

Two weeks before the beginning of the experiments, PDL cells were rapidly thawed in a water bath at 37 °C and placed in culture flasks with Minimum Essential Medium (MEM) (Cultilab, Campinas, SP, Brazil) containing 10% foetal bovine serum (Cultilab) and 1% of penicillin G sodium (10 000 UI), streptomycin (10 mg) and amphotericin B (25 μg) (PSA) (Cultilab). The flasks were then incubated at 37 °C in an atmosphere of 5% CO_2. Cells from passages 5–10 were used.

PDLF (8×10^3 cells per well) were seeded in seven 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 the following solutions ($n = 11$): skimmed pasteurized long-life milk – ultra-high temperature (UHT) (Parmalat, São Paulo, SP, Brazil) (pH 6.8); whole pasteurized long-life milk – UHT (Parmalat) (pH 6.8); Save-A-Tooth's HBSS (Save) (Phoenix-Lazerus) (pH 6.8); recently prepared HBSS (pH 7.0); natural coconut water (pH 5.5); industrialized coconut water (Socôco, Maceió, AL, Brasil) (pH 4.7) and tap water (pH 7.6) (negative control).

For Save, the box of Save-A-Tooth® system (serial 6303, manufacturing date unrevealed, EXP 01/2010) was stored at room temperature, and its solution was used approximately 20 months before the expiration date.

The seven plates were incubated at 5 °C. After 3, 6, 24, 48, 72, 96 and 120 h, the storage media were replaced by MTT solution (1 mg mL^{-1}) (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 (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 a positive control for cell growth.

Statistical analysis

Statistical analysis of the data was accomplished using the Kruskal–Wallis test, complemented by the Scheffé test. Statistical differences were considered significant at $P < 0.05$.

Results

The mean absorbance values, which represent PDL cell viability for each tested medium and for storage periods, are shown in Fig. 1. The Kruskal–Wallis test shows time-dependent results according to the experimental solution analysed ($P < 0.05$).

Minimum essential medium had the greatest capacity to maintain cell viability, and it was significantly better than all other groups ($P < 0.05$). The efficacy of skimmed and whole milk was significantly better than HBSS, Save, industrialized coconut water and tap water at every time period ($P < 0.05$) and from 24 to 120 h was significantly better than natural coconut water ($P < 0.05$). When both skimmed and whole milk were compared, a significant difference was noted only at 120 h, at which skimmed milk showed better results ($P < 0.05$). At 3 and 6 h, there were no significant differences between HBSS, Save and natural coconut water ($P < 0.05$), whereas from 24 to 120 h, Save, industrialized coconut water and tap water were the worst storage media ($P < 0.05$).

Discussion

Although avulsed teeth are generally stored in a medium at room temperature, Sigalas *et al.* (2004) concluded that a lower temperature had a positive effect on cellular viability. Low temperature has the advantage of reducing cellular metabolism (Barile 1994), limiting bacterial growth and preventing milk from souring, which might have an influence on the prognosis of tooth replantation (Ashkenazi *et al.* 1999). This study tested, at 5 °C, the effectiveness of natural and industrialized coconut water, recently prepared HBSS, Save, skimmed and whole milk to maintain the viability of PDLF.

Milk has a physiological osmolality and pH (230–270 mOsm kg^{-1} and 6.5–6.8, respectively) and provides nutrients (Blomlöf 1981) and growth factors to the cells (Belford *et al.* 1995, Gauthier *et al.* 2006). In this study, skimmed and whole long-life milk preserved significantly more viable PDLF than any other experimental solution at every time period. This finding is in agreement with those of Sigalas *et al.* (2004) and Ashkenazi *et al.* (1999), who affirmed that cold milk is suitable for the preservation of the proliferation capacity of PDLF. However, some authors have concluded that milk is effective for only a short period of time (Huang *et al.* 1996). It is likely that methodological differences like the storage temperature and the type of milk used can explain this variation. Huang *et al.* (1996) compared milk at 4 °C and at room temperature with HBSS at 20 °C. In this study, all the experimental storage media were maintained at 5 °C, and long-life pasteurized milk – UHT was used, whilst Huang *et al.* (1996) used regular milk. The UHT pasteurization process is achieved by heating milk at 140 °C for 3 s. This process ensures optimal microbial inactivation, which might prolong its effectiveness in the conservation of cells (Marino *et al.* 2000). Comparing the capacity of regular and long shelf-life milk to preserve the viability of PDLF for up 8 h, Marino *et al.* (2000) did not find differences between them. However, it must be emphasized that the experimental period used was shorter than the one in this study.

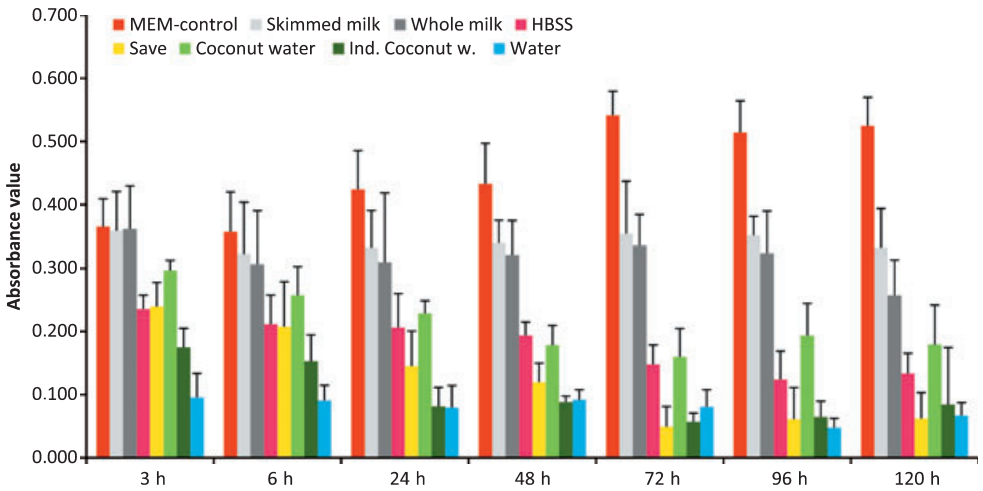

Figure 1 Mean absorbance values, which represent PDL cell viability for each tested media at 5 °C in different periods of time.

When skimmed and whole milk were compared, skimmed milk was found to be better than whole milk only at 120 h. This result is not in agreement with Harkacz *et al.* (1997), who reported that from 3 h, milk with a lower fat content might be more appropriate at maintaining PDL cell viability than would milk with a higher fat content. However, those authors performed their experiments at 37 °C, and this might explain the discrepancies in the findings.

Natural coconut water is sterile and has a 93% water and 5% sugar composition, which gives it a high osmolality. It is rich in proteins, vitamins and minerals such as potassium, calcium and magnesium (Nadanasabapathy & Kumar 1999). According to Gopikrishna *et al.* (2008a,b), this product was better than HBSS and milk in maintaining human fibroblast viability. However, in the present study, it was less effective than skimmed or whole milk. The reason for the differing results is unknown. This discrepancy might be attributed to the different methodologies employed. In their studies, Gopikrishna *et al.* (2008a,b) evaluated, using the trypan blue test, the viability of PDLF after storing freshly extracted teeth in these media at room temperature for 45 min. In this study, the viability was verified by MTT assay after the cells had been stored at 5 °C for 3–120 h.

As natural coconut water is not readily obtained, industrialized coconut water was used. However, the results in this study were disappointing when compared with natural coconut water. It could be hypothesized that its low pH value (4.7) and the presence of other products in its composition, such as acidulants, antioxidants and preservatives, interfered with its performance. This finding is in agreement with a recently published study by Moreira-Neto *et al.* (2009), who verified that coconut water, at 37 °C, was less effective than milk in maintaining human fibroblast viability.

In the present study, HBSS was less effective than skimmed milk, whole milk or natural coconut water. This contradicts the findings of other authors who showed that HBSS was better than milk after 12 h (Hiltz & Trope 1991) or during all experimental period (Huang *et al.* 1996). The reason for the differing results might be attributed to the type of milk and to the temperature of HBSS employed. Whilst Hiltz & Trope (1991) and Huang *et al.* (1996) used regular milk and HBSS at room temperature, UHT long-life milk and HBSS at 5 °C were employed in the present study. The results here obtained seem to suggest that in this lower temperature there are less disposable nutrients from HBSS disturbing cellular metabolism and the reversion of tetrazolium salts in formazan crystals.

In this study, the results were even worse with Save, which was less effective than skimmed milk, whole milk and coconut water for all time periods. At 3, 24 and 48 h, Save performed similarly to HBSS. After 72 h, it was found to be similar to tap water. Other studies carried out with this product revealed similar results (Olson *et al.* 1997, Marino *et al.* 2000, Souza *et al.* 2010a,b). It could be that storage of this product might have influenced the results, because recently prepared HBSS performed better than Save, even though both products have the same components. Recently, Souza *et al.* (2010b) concluded that the storage time of HBSS has a negative influence on its ability to maintain PDLF viability.

Conclusions

Effectiveness of the storage media tested in maintaining PDL cell viability at 5 °C in decreasing order was as follows: skimmed milk and whole milk > natural coconut water > HBSS > Save-A-Tooth® system's HBSS > industrialized coconut water > tap water.

References

American Association of Endodontists (1995) *Recommended Guidelines for the Treatment of Avulsed Permanent Tooth*. Chicago, IL: American Association of Endodontists.

Andreasen JO (1981) Effect of extra-alveolar period and storage media upon periodontal and pulpal healing after replantation of mature permanent incisors in monkeys. *International Journal of Oral & Maxillofacial Surgery* **10**, 43–53.

Ashkenazi M, Sarnat H, Keila S (1999) In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in six different media. *Endodontics & Dental Traumatology* **15**, 149–56.

Ashkenazi M, Marouni M, Sarnat H (2000) In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in four media at room temperature. *Endodontics & Dental Traumatology* **16**, 63–70.

Barile FA (1994) *Introduction to In Vitro Cytotoxicology: Mechanisms and Methods*. Boca Raton, FL: CRC Press.

Belford DA, Rogers ML, Regester GO *et al.* (1995) Milk-derived growth factors as serum supplements for the growth of fibroblast and epithelial cells. *In Vitro Cellular & Developmental Biology* **31**, 752–60.

Blomlöf L (1981) Milk and saliva as possible storage media for traumatically exarticuled teeth prior to replantation. *Swedish Dental Journal* **8**, 1–26.

Blomlöf L, Otteskog P (1980) Viability of human periodontal ligament cells after storage in milk or saliva. *Scandinavian Journal of Dental Research* **88**, 436–40.

Blomlöf L, Lindskog S, Anderson L, Hedström K-G, Hammarström L (1983) Storage of experimentally avulsed teeth in milk prior to replantation. *Journal of Dental Research*, **62**, 912–6.

Doyle DL, Dumsha TC, Sydiskis RJ (1998) Effect of soaking in Hank' balanced salt solution or milk on PDL cell viability of dry stored human teeth. *Endodontics & Dental Traumatology* **14**, 221–4.

Gauthier SF, Pouliot Y, Maubois J-L (2006) Growth factors from bovine milk and colostrum: composition, extraction and biological activities. *Lait* **86**, 99–125.

Gopikrishna V, Thomas T, Kandaswamy D (2008a) A quantitative analysis of coconut water: a new storage media for avulsed teeth. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **105**, 61–5.

Gopikrishna V, Baweja PS, Venkateshbabu N, Thomas T, Kandaswamy D (2008b) Comparison of coconut, water, propolis, HBSS and milk on PDL cell survival. *Journal of Endodontics*, **34**, 587–9.

Harkacz OM, Carnes DL, Walter WA (1997) Determination of periodontal ligament cell viability in the oral rehydration fluid Gatorade and milks of varying fat content. *Journal of Endodontics* **23**, 687–90.

Hiltz J, Trope M (1991) Vitality of human lip fibroblasts in milk, Hank's balanced salt solution and Viaspan storage media. *Endodontics & Dental Traumatology* **7**, 69–72.

Huang SC, Remeikis NA, Daniel JC (1996) Effects of long- term exposure of human periodontal ligament cells to milk and other solutions. *Journal of Endodontics* **22**, 30–3.

Krasner P, Person P (1992) Preserving avulsed teeth for replantation. *Journal of the American Dental Association* **23**, 80–8.

Lekic PC, Kenny DJ, Barrett EJ (1998) The influence of storage conditions on the clonogenic capacity of periodontal ligament cells: implications for tooth replantation. *International Endodontic Journal* **31**, 137–40.

Lindskog S, Blomlöf L, Hammarström L (1983) Mitosis and microorganisms in the periodontal membrane after storage in milk or saliva. *Scandinavian Journal of Dental Research* **91**, 465–72.

Marino TG, West LA, Liewehr FR *et al.* (2000) Determination of periodontal ligament cell viability in long shelf- life milk. *Journal of Endodontics* **26**, 699–702.

Moreira-Neto JJS, Gondim JO, Raddi MSG, Pansani CA (2009) Viability of human fibroblasts in coconut water as a storage medium. *International Endodontic Journal* **42**, 827–30.

Nadanasabapathy S, Kumar R (1999) Physicochemical constituents of tender coconut (cocos nucifera) water. *Indian Journal of Agricultural Sciences* **69**, 750–1.

Olson BD, Mailhot JM, Anderson RW, Schuster GS, Weller RN (1997) Comparison of various transport media on human periodontal ligament cell viability. *Journal of Endodontics* **23**, 676–9.

Sant'ana ACP, Marques MM, Barroso EC, Passanezi E (2002) Cultura e caracterização de células derivadas de ligamento periodontal humano. *Revista da Faculdade de Odontologia de Bauru* **10**, 134–40.

Schwartz O, Andreasen FM, Andreasen JO (2002) Effects of temperature, storage time and media on periodontal and pulpal healing after replantation of incisors in monkeys. *Dental Traumatology* **18**, 190–5.

Sigalas E, Regan JD, Kramer PR, Witherspoon DE, Opperman LA (2004) Survival of human periodontal ligament cells in media proposed for transport of avulsed teeth. *Dental Traumatology* **20**, 21–8.

Souza BDM, Lückemeyer DD, Felippe WT, Simões CMO, Felippe MCS (2010a) Effect of temperature and storage media on human periodontal ligament fibroblast viability. *Dental Traumatology* **26**, 271–5.

Souza BDM, Bortoluzzi EA, Teixeira CS, Felippe WT, Simões CMO, Felippe MCS (2010b) Effect of HBSS storage time on human periodontal ligament fibroblast viability. *Dental Traumatology* Published online ahead of print. DOI: 10.1111/j.1600-9657.2010.00933.x.

Trope M, Friedman S (1992) Periodontal healing of replanted dog teeth stored in Viaspan, milk and Hanks' balanced salt solution. *Endodontics & Dental Traumatology* **8**, 183–8.