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Fibroblast Viability after Storage at 20 °C in Milk, Hank's Balanced Salt Solution and Coconut Water

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The objective of this study was to evaluate the effectiveness of various storage media at 20 °C in maintaining the viability of human periodontal ligament fibroblasts (HPLF) over time. HPLF were maintained at 20 °C in skim milk (SM), whole milk (WM), freshly prepared Hank's balanced salt solution (HBSS), Save-A-Tooth®, natural coconut water (NCW), coconut water industrialized (ICW) and tap water (negative control) for 3, 6, 24, 48, 72, 96 and 120 h. Cells maintained in Minimal Essential Medium (MEM-37) at 37 °C served as a positive control. Cell viability was determined by MTT assay. Statistical analysis was performed by Kruskal-Wallis test and Scheffe test (α = 5%). From 24 h, NCW was significantly better in maintaining cell viability than all other tested storage media ($p<0.05$). SM and WM were significantly better than HBSS for up to 72 h. Save-A-Tooth® and ICW were the worst conservation storage media. In conclusion, the effectiveness of the tested storage media to maintain the viability of the periodontal ligament cells was as follows, in a descending order: NCW > MEM-37> SM and IM> HBSS> ICW > Save-A-Tooth®> tap water.

Key Words: periodontal ligament, fibroblasts, storage media, Milk, HBSS, Coconut Water

Introduction

Tooth avulsion is characterized by complete displacement of a tooth from its alveolar socket. During the extraalveolar period, periodontal cells are subject to contamination and dehydration and may become necrotic (1). The success of tooth replantation could be improved if the tooth is replanted immediately (1,2). However, as immediate repositioning is not always possible, it is extremely important to choose a suitable storage medium for maintenance of the periodontal ligament fibroblasts (PDLF) viability (1,2).

Hank's balanced salt solution (HBSS), available in the Save-A-Tooth® system (Phoenix-Lazerus, Shartlesville, PA, USA), has been recommended as the storage medium to maintain PDLF viability (1). Some experiments have shown that it is a suitable medium for the storage of avulsed teeth for periods ranging from 3 to 72 h (2-7). However, a disadvantage of this solution is that it may not be readily available in locations where tooth avulsion is likely to occur.

Milk is another extensively studied storage medium, which has gained wide acceptance (3-12). Some studies have reported that its effectiveness can last from 3 h to 48 h (4-8,11-13). Although there has been some controversy (12,13), it has been suggested that milk with a lower fat content might be more appropriate in maintaining PDLF viability than milk with a higher fat content (14).

Coconut water is another alternative storage medium and has been recently studied (7,13,15-17). It has almost the same isotonicity as sports drinks and has been used for rehydration and salt replacement. It is available in its natural form directly from the coconut or in long shelf-life packages (industrialized form), mainly in tropical countries (15). Coconut water has been studied as a storage medium, but with controversial results (7,12,13,15-18). While some studies reported that coconut water was superior to HBSS and milk in maintaining PDLF viability (13,15), others found that it was worse than milk (7,12,18).

It should be noted that these studies used different types of coconut water at different temperatures. One study used natural coconut water at room temperature (15), while another was done at 5 °C (12). Other studies used long shelf-life coconut water at 37 °C (7,18) and at 5 °C (12), while yet another study used long shelf-life coconut water with pH adjusted to 7 at 4 °C (13).

Recently, the effectiveness of all these storage media for maintaining the PDLF viability was evaluated at 5 °C (12), because some studies reported that lower temperatures have the advantage of reducing cellular metabolism (19) and limiting bacterial growth (3). Considering that avulsioned teeth are generally maintained in a solution at room temperature, the purpose of this study was to compare, at 20

°C, the effectiveness of SM, WM, HBSS, Save, NCW and ICW in maintaining PDLF viability over time, by the analysis of cell metabolism using MTT assay. The study hypotheses were that (I) HBSS and Save would exhibit better performance in maintaining cell viability over the short and long term than SM, WM, NCW and ICW; (II) NCW would exhibit better performance in maintaining cell viability than ICW; (III) SM would exhibit better performance in maintaining cell viability than WM; (IV) HBSS and Save are similar in keeping the cells viable over the short and long term; and (V) Storage room temperature has a positive impact on the ability of storage solutions to maintain the viability of PDLF.

Material and Methods

The project was approved by the Ethics Committee for Human Research. Procedures for the primary culture and establishment of the cell strain were carried out according to a technique described by Souza et al. (11).

For the experiments, PDLF were cultured in 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) (PSA) (Cultilab). Flasks were incubated at 37 °C in an atmosphere of 5% CO_2. Cells from passages 5 to 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 UHT (ultra-high temperature, long life) pasteurized milk (SM) (Parmalat, São Paulo, SP, Brazil) (pH 6.8); whole UHT (ultra-high temperature, long life) pasteurized milk (WM) (Parmalat) (pH 6.8); freshly prepared HBSS (HBSS) (pH 7.0); Save-A-Tooth's HBSS (Save) (Phoenix-Lazerus, Shartlesville, PA, USA) (pH 6.8); natural coconut water (NCW) (pH 5.5) and industrialized coconut water (ICW) (Socôco, Maceió, AL, Brazil) (pH 4.7). Tap water (water) (pH 7.6) was used as negative control and MEM at 37 °C as a positive one. Plates were incubated at 20 °C and after 3, 6, 24, 48, 72, 96 and 120 h the storage media were replaced by MTT solution (1 mg/mL) (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 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

Statistical analysis of the data was accomplished using the Kruskal-Wallis test, complemented by the Scheffé test. Statistical differences were considered significant at $p<0.05$. Tests were performed using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) software.

Results

The mean absorbance values, which represent PDLF viability for each tested medium and for storage periods are shown in Figure 1. The Kruskal-Wallis test showed time-dependent results according to the analyzed experimental solution ($p<0.05$).

SM and WM were the best storage media after 3 h, followed by NCW, HBSS, Save and ICW. At 6h, the efficacy of NCW was similar to WM and SM and superior to HBSS, Save and ICW. From 24 h and onwards, NCW was significantly better than all other media, and Save, ICW and water were the worst storage media ($p<0.05$). At 48 and 72 h, SM and WM were found significantly better than HBSS, Save, ICW

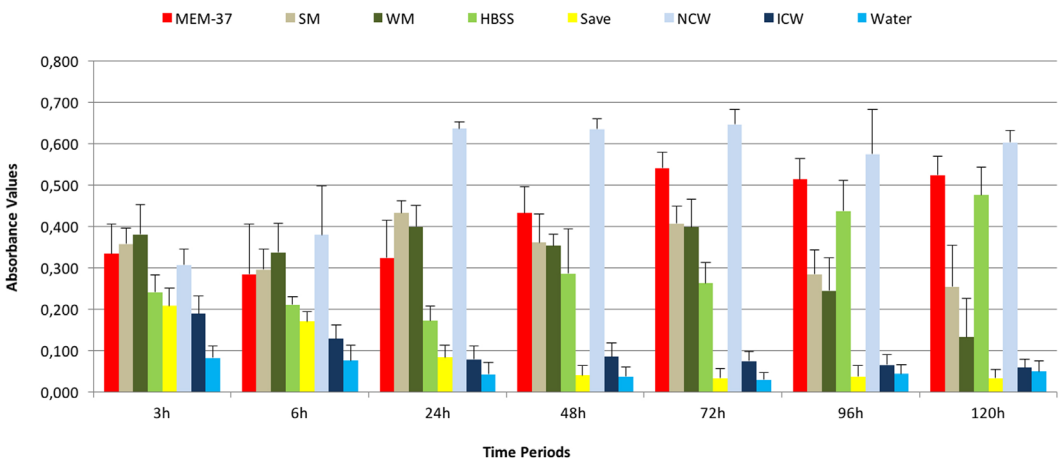

Figure 1. Mean absorbance values, which represent PDL cell viability for each tested media at 20 °C in different periods of time. MEM-37, Minimum essential medium at 37 °C; SM, skimmed Milk; WM, Whole Milk; HBSS, recently prepared Hank's balanced salt solution; Save, Save-A-Tooth® system's HBSS; NCW, natural coconut water; ICW, industrialized coconut water; water, tap water.

and water ($p<0.05$). At 96 and 120 h, HBSS was found significantly better than SM and WM ($p<0.05$). When both SM and WM were compared, significant differences were found only at 96 and 120 h, where SM showed better results ($p<0.05$).

Discussion

The first tested hypothesis was partially rejected because from 24 h onwards, NCW was significantly better than all other media for maintaining cell viability. SM and WM were found significantly better than HBSS for up to 72 h and Save and ICW were the worst storage media. Studies carried out to evaluate different storage media have demonstrated that the time of effectiveness differs according to the medium (12). Previous studies have shown that milk is effective for a period between 3 h and 48 h (4,5,7,11-13), whereas HBSS and coconut water can maintain the cell viability for up to 72 h (5) and 24 h (8,12,13,16), respectively.

The results of NCW in the present study were in accordance with previous studies (13,15), which showed that the efficacy of coconut water was better than milk and HBSS. However, they are also in contrast to other studies (7,12,18), whose results showed that industrialized coconut water at 37 °C (7,18), and natural and industrialized coconut water at 5 °C (12), were worse than milk in maintaining PDL cell viability. The different results in the experiments may be attributed to the type of used cells, type of coconut water and methodological differences. Coconut water, a natural sterile product, has 93% water and 5% sugar in its composition, which gives it a high osmolality. It is rich in proteins, vitamins and minerals (15). However, it has an acid pH (5.5), which is deleterious to cell metabolism (18), and makes its favorable performance questionable in this study. It is possible that the results obtained with coconut water may be attributed to the presence of a white deposit observed after 24 h at the bottom of the well filled with this medium. This deposit became more noticeable over time, which may have further interfered in the optical density readings. Previous studies have stated that colored substances can interfere in the absorbance readings, generating higher values (20). Perhaps the procedure of filtering the coconut water before putting it into contact with the cells, along with the act of washing the cavity with PBS before placing the MTT, could prevent the formation of the deposit, or also its removal, which may have overestimated the results. A study is being conducted to evaluate this.

Since natural coconut water is not readily obtained, industrialized coconut water was also tested. The second hypothesis was accepted because NCW had better performance than ICW, which was similar to water. The present finding was in accordance with other studies (7,12). Probably, its low pH (4.7) and the presence of other products such as acidulants, antioxidants and preservatives in its composition, may have affected its performance. Previous studies showed that industrialized coconut water, with pH adjusted to 7 using triethanolamine, had performance similar to milk (17), and better than milk and HBSS (13). The pH adjustment could inhibit inflammation in human skin (21) and is essential for ensuring the adequate performance of both natural and industrialized CW (13,18).

The third hypothesis was partially accepted because SM had better performance than WM from 96 to 120 h, agreeing with Souza et al. (12). However, the other study showed that WM performed better than SM at 24 h to preserve cell viability (13). These results are in disagreement with the previous study (14) which affirmed that until 3 h, milk with a lower fat content could be more appropriate for maintaining PDL cell viability than milk with a higher fat content. However, these authors used regular milk and performed the experiments at 37 °C, explaining the discrepancies of the findings. In the current study, skimmed milk and whole milk were effective in preserving the viability of PDLF for up to 48 h. After 72 h, their effectiveness decreased considerably. It has been hypothesized that the pH in milk decreases over time, creating an unsuitable environment for cell survival (11,22). SM and WM had better performance than HBSS for up to 72 h. This finding is consistent with other recent reports, which showed that the cell viability observed after contact with milk over 24 h was superior to the HBSS (4,13). However, it was also in contrast to previous studies, which had reported better (2,3,5) or similar (7,23) effectiveness of HBSS in relation to milk. It is likely that methodological differences such as storage temperature, experimental period and type of milk can explain this variation. While long-life pasteurized milk - UHT (ultra-high temperature) was used in this experiment, the others 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 may extend its effectiveness for the conservation of cells (10). Another possible explanation for the positive results obtained with milk is that, besides the physiological pH and osmolality and the presence of some nutrients (3), it contains growth factors (24) such as EGF, BTC, IGF-I, IGF-II, TGF-β1, TGF-β2, FGF1, FGF2 and PDGF (24).

The fourth hypothesis was partially rejected because HBSS and Save are similar in keeping the cells viable only at 3 and 6 h. After 48 h, Save was found to be similar to tap water. Other studies carried out with the HBSS present in the Save-A-Tooth® system box revealed similar results (10,12,25). In the current study, Save was used approximately 7 months after its acquisition. The time elapsed after preparation may have influenced negatively the results (12,25). Save storage may cause alterations in the concentrations of

its components, making this solution unable to nourish adequately the cells for more than 6 h (25).

The fifth hypothesis was partially accepted because storage room temperature (20˚C) had a positive impact on the ability of NCW and HBSS to maintain the viability of PDLF. From 96 h onward, HBSS had better performance than SM and WM and similar to MEM-37, disagreeing with the other study at 5 °C (12). It could be speculated that there are more available nutrients from HBSS at room temperature, maintaining cellular metabolism and the reversion of tetrazolium salts in formazan crystals. Another study showed that as long as the PDLF are stored in an appropriate medium such as HBSS, DMEM and milk, the temperature plays only a minor role (8). However, it must be emphasized that the experimental period used was shorter than the one in this study.

In some cases, patients have more serious injuries than dental avulsion, remaining at the hospital, unable to receive appropriate dental care. In these situations, the avulsed teeth should be stored in a physiological environment for a few days until replantation. Thus, this study was carried out taking into consideration storage periods from 3 to 120 h in order to be clinically relevant.

Under the tested condition, the effectiveness of the storage media tested in maintaining PDLF viability at 20 °C in decreasing order was as follows: CNW > MEM-37 > SN and WM > HBSS > ICW > Save-A-Tooth® > water.

Resumo

O objetivo deste estudo foi avaliar a efetividade de vários meios de conservação a 20 °C em manter a viabilidade de fibroblastos do ligamento periodontal humano (FLPH) ao longo do tempo. FLPH foram conservados a 20 °C em leite desnatado (LD), leite integral (LI), solução salina balanceada de Hank (HBSS) recém preparada, Save-A-Tooth® (Save), água de coco natural (ACN), água de coco industrializada (ACI) e água de torneira (água - controle negativo) por 3, 6, 24, 48, 72, 96 e 120 h. Células conservadas em Meio Essencial Mínimo (MEM-37) a 37 °C serviram como controle-positivo. A viabilidade celular foi determinada pelo ensaio MTT. A análise estatística dos dados foi realizada pelos testes Kruskal-Wallis e Scheffé (α=5%). A partir de 24 h, ACN foi significantemente melhor em manter a viabilidade celular do que todos os outros meios testados ($p<0,05$). LD e LI foram significantemente melhores do que a HBSS por até 72 h. Save e ACI foram os piores meios de conservação. Concluindo, a efetividade dos meios de conservação testados em manter a viabilidade das células do ligamento periodontal foi a seguinte em ordem decrescente: ACN > MEM-37 > LD e LI > HBSS > ACI > Save > água.

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