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Effect of HBSS storage time on human periodontal ligament fibroblast viability

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Abstract – Hank's balanced salt solution (HBSS) is recommended for the storage of avulsed teeth. The objective of this study was to evaluate if the HBSS storage time influences its ability to maintain the viability of human periodontal ligament fibroblasts (PDLF) by the analysis of cell metabolic function using MTT assay. PDLF were kept at 20°C for 3, 6, 24, 48, 72, 96 and 120 h in recently prepared HBSS (HBSS), HBSS stored for 6 months (HBSS 6 M), HBSS stored for 12 months (HBSS 12 M), and in Save-A-Tooth system's HBSS (Save). Minimum essential medium (MEM) at 37°C and tap water at 20°C served as positive and negative controls, respectively. Cell viability was determined by the tetrazolium salt-based colorimetric (MTT) assay. Data were statistically analyzed by the Kruskal–Wallis and Scheffé tests ($\alpha = 5\%$). Starting with the 6 h time-point, HBSS was significantly more effective than HBSS 6 M, HBSS 12 M and Save in maintaining cell viability. HBSS 6 M effectiveness was similar to that of HBSS 12 M for up to 48 h, becoming higher at 72 h. In conclusion, the storage time of HBSS had a negative influence on its ability to maintain PDLF viability.

Several experiments have been carried out in an attempt to find the ideal storage medium for avulsed teeth (1–4). Hank's balanced salt solution (HBSS) (5) and other protective (6, 7) medium containing a pH-balanced cell culture fluid have been recommended for this purpose. HBSS is composed of essential cell nutrients, has a pH of 7.2 and an osmolality of around 320 mOsm kg^{-1} (8), and is able to keep cells morphologically normal for up to 72 h (2). Huang et al. (9) showed that HBSS was better than milk, maintaining 46.8% of the cells viable for 72 h. In relation to maintaining cell viability, and clonogenic and mitogenic capacity, HBSS has generated better results when compared with conditioned medium, Viaspan® (10), and minimum essential medium (MEM) (11).

HBSS can be manipulated according to the formula described by Krasner & Person (8). For the conservation of avulsed teeth, it is commercially available in the Save-A-Tooth® system (Phoenix-Lazerus, Shartlesville, PA, EUA). Some studies with cell culture have shown that the HBSS contained in the Save-A-Tooth® system is less effective than milk (4, 12, 13) and freshly prepared HBSS (4), and is similar to tap water after 24 h of contact (4). In the study by Souza et al. (4), HBSS was manipulated using the same components as HBSS in the Save-A-Tooth® system, but in concentrations similar to those mentioned by Krasner & Person (8), as the concentration of each component is not specified by the manufacturer of Save-A-Tooth® system. It is known that the storage period of a product can affect its effectiveness. In that study (4), the HBSS of Save-A-Tooth® system was used approximately 6 months after its acquisition. According to the authors, it is possible that storage of this product might have negatively influenced the results, as HBSS used immediately after its preparation had a better performance than Save-A-Tooth system' HBSS. The objective of this study was to address this issue, evaluating if the storage time of HBSS influences its ability to maintain periodontal ligament fibroblasts (PDLF) viability by the analysis of cell metabolic function using MTT assay.

Materials and methods

Procedures for the primary culture and establishment of the cell strain were carried out according to the technique described by Sant'ana et al. (14) and modified by Souza et al. (4).

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 MEM (Cultilab, Campinas, SP, Brazil) containing 10% fetal 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 (Fanem, HF 212, São Paulo, SP, Brasil). The cells were subcultured every 4 days until an adequate number of cells were obtained. 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 ($n = 11$): recently prepared HBSS (HBSS) (pH 7.2), HBSS stored for 6 months (HBSS 6 M) (pH 7.0), HBSS stored for 12 months (HBSS 12 M) (pH 7.0), Save-A-Tooth system's HBSS (Save) (Phoenix-Lazerus) (pH 7.0), and tap water (pH 7.6) (negative control). The absorbance values, after cells had been stored in MEM at 37°C, were used as a positive control for cell growth.

HBSS was prepared according to the Save-A-Tooth® manufacturer's formula: sodium chloride (8 g l^{-1}), D-glucose (0.4 g l^{-1}), potassium chloride (0.4 g l^{-1}), sodium bicarbonate (0.35 g l^{-1}), sodium phosphate (0.09 g l^{-1}), potassium phosphate (0.14 g l^{-1}), calcium chloride (0.14 g l^{-1}), and magnesium sulfate (0.1 g l^{-1}). The concentrations were based on formula presented by Krasner & Person (8).

For Save, the box of Save-A-Tooth® system was stored at room temperature and its solution was used approximately 1 month after its acquisition. (serial 8231, manufacturing date unrevealed, EXP 01/2012).

The seven plates were incubated at 20°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 dimethylsulfoxide (DMSO) was added to the wells. Cell viability was determined by measuring the optical density at 540 nm on a spectrophotometer (Labsystems Multiskan MS 352, Haverhill, MA, 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 mean absorbance values that represent PDLF viability for each media, and the storage periods tested are shown in Fig. 1. The Kruskal–Wallis test showed time-dependent results, according to the experimental solution analyzed ($P < 0.001$).

At any of the time-periods analyzed, the least effective medium was tap water. At 3 h, there was no difference between the experimental solutions and MEM ($P > 0.05$). From 6 h onwards, MEM, followed by HBSS, showed greater ability to maintain cell viability than the other media ($P < 0.001$). The effectiveness of HBSS 6 M was similar to that of HBSS 12 M for up to 48 h ($P > 0.05$), becoming higher at 72 h ($P < 0.001$). From 24 h onwards, Save was as effective as tap water ($P > 0.05$).

Discussion

HBSS is a common medium used in biomedical research involving different types of cells (8). Several studies have shown that this solution is an effective medium for the conservation of avulsed teeth (1, 2, 15), because it has the ability to preserve and reconstitute the periodontal ligament cells of teeth maintained out of the alveolus for a long period of time (8). According to Krasner (16), HBSS does not require refrigeration, and is valid for 2 years. Its major drawback is not being available in places where avulsion commonly occurs. However, with the commercialization of HBSS in the early 1990s (Save-A-Tooth® system), it has become easier to purchase the product. Some studies with cell culture have produced disappointing results with the use of Save-A-Tooth system' HBSS (4, 12, 13). After 8 h of contact, this product was less effective than whole (12, 13) and long-life milk (13), but similar to Gatorade® (12). Souza et al. (4) found that after 24 h of contact, the results obtained with Save-A-Tooth system' HBSS were much less satisfactory than those obtained from newly developed HBSS. Considering that these products have the same components, the authors suggested that the poor performance of Save-A-Tooth system' HBSS may have been caused by its storage period (6 months), or by the possibility that the concentration of its components were quite different from those used for HBSS manipulation. The present study was conducted to evaluate if the HBSS storage time influences its ability to maintain PDLF viability by the analysis of cell metabolic function using MTT assay.

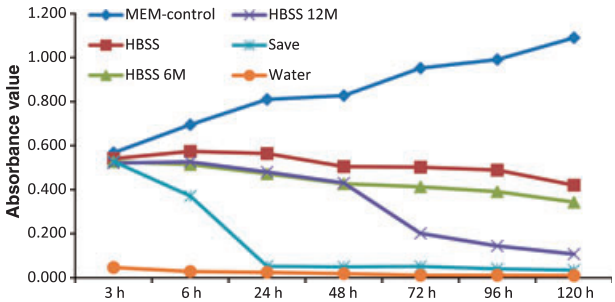

Fig. 1. Viability, expressed as absorbance values, of periodontal ligament fibroblasts conserved at 20°C using different media and time periods.

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a hydrosoluble compound easily incorporated by viable cells, and reduced in the metabolic by the action of dehydrogenases. On reduction, MTT is converted into formazan crystals, which are stored in the cellular cytoplasm and later solubilised by dimethylsulfoxide (DMSO). Cell viability and metabolic activity are quantified by the amount of formazan produced, which is expressed as an absorbance value, analyzed by spectrophotometry (17–19).

Avulsed teeth are normally not replanted at the site of the accident (20). 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 physiological environment for a few days until replantation. Thus, this study was carried out considering storage periods from 3 to 120 h to be clinically relevant.

The results revealed that at 3 h, there was no difference between the experimental solutions and MEM ($P > 0.05$). However, starting from 6 h, HBSS was significantly more effective when compared with HBSS 6 M and HBSS 12 M, both of which showed a similar performance for up to 48 h. From 72 h onwards, the effectiveness of HBSS 12 M declined considerably. These findings suggest that HBSS storage times of 6 and 12 months cause an alteration in the concentration of its components, making them insufficient to nourish the cells for more than 6 h. Given the limitations of cell culture studies, clinical studies should be conducted to confirm these results.

Regarding Save, the results of this study confirm those of previous studies (4, 12, 13). Although it was effective during the first 3 h, its performance declined from 6 h onwards, becoming similar to that of tap water after 24 h of contact. It is important to highlight that although Save had been stored at room temperature for only 1 month before being used, its effectiveness was significantly lower than HBSS stored for 6 and 12 months. This suggests, as previously stated by Souza et al. (4), that there are differences in the concentrations of the components used for manufacturing the HBSS of Save-A-Tooth® system and the HBSS employed in this study.

According to the results obtained, it is concluded that the storage time of HBSS has a negative influence on its ability to maintain PDLF viability.

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