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Comparison of soymilk, powdered milk, Hank's balanced salt solution and tap water on periodontal ligament cell survival

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Abstract – The purpose of this study was to evaluate the ability of soymilk, powdered milk, and Hank's balanced salt solution (HBSS) to maintain human periodontal ligament (PDL) cell viability *in vitro*. PDL cells were obtained from extracted healthy third molars and cultured in Dulbecco's modified Eagles medium (DMEM). The cultures were exposed for 1, 2, 4, and 8 h to experimental solutions (tap water served as negative control and DMEM as positive control) at 37°C. The viable cells were then counted using the trypan blue exclusion technique. Data were analyzed by using one-way ANOVA, *post hoc* Scheffe and two-way ANOVA test. Statistical analysis showed that HBSS, powdered baby formula, and soymilk maintain cell viability equally well in different periods of times. Tap water cannot keep cells viable as well as other solutions. Soymilk and powdered baby formula can be recommended as suitable storage media for avulsed teeth for up to 8 h.

When avulsion occurs, rupture of neurovascular bundle leads to loss of pulp vitality and within a short time period (1), the periodontal ligament cells begin to necrose. Necrosed periodontal ligament (PDL) cells in replanted tooth promote inflammatory process and in severe situations lead to replacement root resorption and certainly loss of tooth (2).

Soder et al. (3) showed that the number of necrosed PDL cells increase by more extra oral dry time, and after 2 h, it was not possible to detect cell viability. So ideal treatment is quick replantation of avulsed tooth to prevent more cell necrosis and replacement root resorption (4). Unfortunately, some conditions such as lack of knowledge in public usually postpone the immediate replantation. In such situations, the tooth should be kept in suitable storage media to preserve maximum PDL cells viability before replantation (5).

Hank's balanced salt solution (HBSS) is recommended as storage media of choice by AAE (5). It has appropriate pH and osmolality to maintain PDL cell viability (6–10). HBSS is non-toxic and contains many essential nutrients (9, 11). As HBSS is not available to the public, several investigations have been carried out to identify the suitable alternative media. It has been shown that physiologic osmolality is an important factor of transport media and with values above 230 mOsm μg^{-1} provides more optimal results (6, 12, 13).

Tap water, because of its hypotonicity, is considered unsuitable media and causes rapid cell lysis (14).

Several *in vivo* and *in vitro* investigations have indicated the efficacy of milk in maintaining PDL cell viability and suggested it as suitable storage media (15–17). Appropriate osmolality and pH for optimal growth of cells accompanied by the presence of nutritional substances in milk may be responsible for its acceptable results as storage media (15, 16).

The regular pasteurized milk has the advantage of lower bacterial content than bovine milk because of its pasteurization process. As regular milk has short life and needs refrigeration, Marino et al. (18) examined long shelf-life milk to overcome these disadvantages. They mentioned that at 8 h, both regular and long shelf-life milk showed better PDL cell viability than save-A-tooth.

Harkacz et al. (19) suggested that regular milks with lower fat content may be more capable for maintaining PDL cell viability than milks with higher fat content. In an *in vitro* study, Pearson et al. (15) tested the efficacy of several milk substitutes in maintaining PDL cell viability in comparison with regular pasteurized whole milk. They indicated that reconstituted powdered milk, Enfamil powder baby formula, was more effective storage media than regular milk for at least 4 h.

Recently, an *in vivo* study indicated similar results from reconstituted powdered milk and long shelf-life milk when used as storage media for avulsed teeth (15). Powdered milk can be transported easily, do not need any special conservation, and have a long shelf-life (15, 18).

The efficacy of powdered milks as storage media is not similar in all types. Blomlof (6) showed that a Swedish milk called 'film jolk' could not effectively maintain cell viability even at 1-h time period.

Taking the properties of powdered milk into consideration, it was hypothesized that it could be a storage media for transportation of an avulsed tooth. Currently, no studies have suggested using soymilk as storage media. Soymilk is a beverage made from soybeans. It is long shelf life milk that has many nutrients for maintaining the viability of PDL cells and for nourishing them. It contains approximately the same proportions of proteins as cow's milk. It is a good substitute for milk for people who are lactose intolerant and allergic to proteins of cow milk. It has no cholesterol, and the amount of fat is very low in it. It is a good source of minerals and vitamins as well.

The aim of this study was to determine the efficacy of soymilk in comparison with reconstituted powdered milk (Humana powdered baby formula) and HBSS in maintaining PDL cell viability.

Materials and methods

Human PDL fibroblasts used in this study were obtained from healthy third molar teeth. In the manner described by Mailhot et al. (20), the isolation and preparation of the primary cell cultures were accomplished. The teeth were washed in sterile saline solution to eliminate the residual blood. The PDL samples were scraped with a sterile scalpel, and the scrapings were placed into a 35-mm culture dish. The explants were incubated with Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus penicillin 100 u ml^{-1}, streptomycin 100 mg ml^{-1}, and amphotericin 2.5 mg ml^{-1}. The culture dishes were incubated at 37°C in an atmosphere of 5% CO_2. After 1 week of incubation, cells were fed every other day until cells reached confluency. For subcultivation, cells were detached by trypsin treatment and passaged into 25-cm^2 tissue culture flasks. When cultures reached confluency, they were transferred to 75-cm^2 flasks. Once confluent, 4th–7th passage cells were detached using trypsin. The cell suspension was centrifuged for 10 min at 130 ***g*** at 25°C, the supernatant fluid was drawn off, and the cells were resuspended in DMEM containing 10% FBS and antibiotic. Cells were then counted using a hemocytometer, and the average of the two readings was used to determine the number of cells per milliliter. For the experiment, 24-well plastic tissue culture plates were plated with 1 ml of medium containing $\sim 8 \times 10^4$ cells ml^{-1} in each well. The plates were then incubated at 37°C in 5% CO_2 for 24 h to allow the cells to attach to the plastics. On the day of treatment, the culture medium was drained from each well, the cells were rinsed three times with 1 ml of sterile phosphate-buffered saline (PBS), and the cells were exposed to 1 ml of the different experimental solutions. The storage solutions used in the experiments were as follows: (i) tap water as the negative control, (ii) DMEM containing 10% FBS and antibiotics as the positive control, (iii) HBSS (Biosera-XC-S2065; Biosera Ltd., Ringmer, East Sussex, UK), (iv) Humana powdered baby formula (Humana GmbH, Herford, Germany) prepared according to the manufacturer's instruction (five full scoops dissolved in 150 ml of previously boiled tap water) and (v) soymilk (Maxoy, Tehran, Iran). The plates were incubated at 37°C in 5% CO_2 for time periods of 1, 2, 4, or 8 h. At the appropriate times, the medium was removed from the wells and washed three times with 1 ml sterile PBS. Then trypsin was added, and the plates were incubated at 37°C for 5–10 min. Full-growth medium (50 μl) and 0.4% trypan blue (50 μl) were added to each well, and the plates were returned to the incubator for another 5 min. After this time, a (50 μl) aliquot was removed and placed under a coverslip on a hemocytometer, and both the viable and the non-viable cells were counted under the microscope (Olympus-ChX11; Olympus, Tokyo, Japan) with magnification of 10×10.

pH measurements were performed on the test solutions using a Fisher Accumet pH meter model b25 MP. Osmolality measurements were obtained with Osmomat 030 (Gonotec, Berlin, Germany) calibrated from 100 to 500 mOsm kg^{-1}. The osmolality of soymilk was 264 mOsm and for powdered milk was 259 mOsm, when it is dissolved in previously boiled tap water.

The results were statistically analyzed using SPSS 19 software program (SPSS Inc., Chicago, IL, USA). Data were statistically analyzed with two-way ANOVA test to assess the effects of different media (groups) and time intervals on cell viability. A *post hoc* Scheffe test was then used to evaluate the probable differences among groups. For comparing the groups more details, one-way ANOVA test is employed in each time interval. A level of $P < 0.05$ was accepted as statistically significant.

Result

Figure 1 shows the ability of different media in preserving cell viability in different times. Two-way ANOVA test showed that there were significant differences among the media at all time periods. Results of Scheffe test indicated that all the experimental media performed

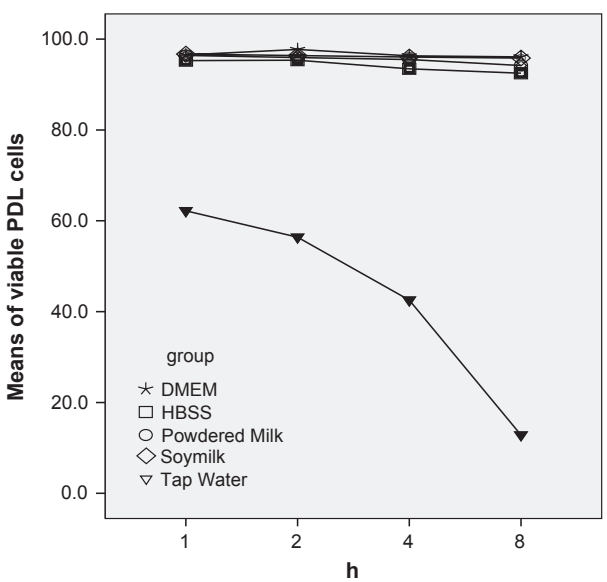

Fig. 1. Mean percentage of viable periodontal ligament cells.

better than water in maintaining PDL cell viability. Results of one-way ANOVA test show that HBSS, powdered milk, and soymilk preserve cell vitality as good as positive control (DMEM) at all experimental time periods. All experimental media maintain PDL cell viability up to 8 h, expectation is water.

Discussion

As we just aimed to determine the viability of PDL cells in experimental solutions, we chose the Trypan blue exclusion staining technique. This technique is quick, easily performed and distinctively differentiates non-viable cells from viable cells. However, the health of the PDL cells and their ability to proliferate cannot be determined from this technique; but determination of the health of the PDL cells was not the aim of this study.

We used human PDL fibroblasts as source of cells in this study which were obtained from clinically healthy extracted third molar teeth. In some previous investigations (9, 21), fibroblasts were obtained from sources other than the PDL-like lip or gingiva. Although the morphology of these fibroblasts is similar, their characteristics in culturing are different. Fibroblasts obtained from PDL reflect more closely the ability of cells to remain viable in culture (9).

The results of this study showed that soymilk and powdered milk are effective storage media for avulsed teeth as HBSS. HBSS is a standard saline solution, which is widely used in biomedical research to support the growth of many cell types. It is non-toxic, pH-balanced, and contains many essential nutrients. HBSS has an osmolality that ranges from 270 to 320 mOsm (9, 11). Tap water is hypotonic and is not a suitable storage medium. It has been shown that solutions with osmolality in a range of 230–400 mOsm are good for cell growth, and optimal growth will occur in a range of 290–330 mOsm (13, 22). The osmolality of soymilk is 264 mOsm and of powdered milk is 259 mOsm when it is dissolved in previously boiled tap water.

Physiological pH is another critical factor for PDL cells viability. It has been indicated that cells can survive between pH 6.6 and 7.8 (23). All of the solutions investigated in our study have physiologic pH. The nutritive value of soymilk and powdered milk may be another factor that explain why they preserve PDL cells viable similar to HBSS.

Recently, Pearsson et al. (15) indicated that powdered milk, Enfamil powder baby formula, is an effective storage medium in maintaining PDL cells viable. Our study on Humana baby formula confirmed powdered milk as a suitable storage media. Powdered milk Humana has suitable pH and osmolality and nutritive value. Powdered milk can be transported easily, does not need any special conservation, and have a long shelf-life.

Soymilk is a kind of long shelf-life milk that is rich in many essential amino acids. The great advantage of this milk is that unlike cow's milk, it has little saturated fat and no cholesterol. Maintenance of viability of PDL cells in the soymilk may be due to the nutrients that are present in soymilk such as proteins, essential amino acids, vitamins and minerals which help in nourishing the cells and maintaining their viability.

Based on the favorable results obtained in this study, soymilk and powdered milk Humana can be recommended as a suitable storage media for avulsed teeth. Ease of storage and long shelf-life make these suitable for schools, gyms, and outdoor athletic fields, where tooth avulsions are most likely to occur.

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