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Viability of human fibroblasts in coconut water as a storage medium

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Abstract

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Aim To evaluate the effectiveness of a new storage medium for avulsed teeth, coconut water, in maintaining the viability of human fibroblasts.

Methodology Cell viability after different time periods was evaluated in the following storage media: coconut water, coconut water with sodium bicarbonate, milk, saline and still mineral water. Human fibroblasts were seeded in Eagle's minimal essential medium (EMEM) supplemented with 7.5% foetal calf serum. After trypsinisation, 100 μL of culture medium containing approximately 10^4 cells mL^{-1} were collected and pipetted into the wells of 96-well plates, which were incubated overnight in 5% CO_2 and 95% air mixture at 37 °C. EMEM was then replaced by the storage media and the plates were incubated at 37 °C for 1, 2 and 4 h. Cell viability was determined using the neutral red assay. The proportions of viable cells after exposure to the storage media were analysed statistically by ANOVA and the least significant difference (LSD) test ($\alpha = 5\%$).

Results Milk had the greatest capacity to maintain cell viability ($P < 0.05$), followed by coconut water with sodium bicarbonate and saline. Coconut water was significantly worse at maintaining cell viability compared to milk, coconut water with sodium bicarbonate and saline. The smallest number of viable cells was observed for mineral water ($P < 0.05$).

Conclusion Coconut water was worse than milk in maintaining human fibroblast cell viability.

Keywords: avulsion, coconut water, human fibroblasts, storage media.

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Introduction

The reported incidence of tooth avulsion is approximately 16% of all traumatic injuries to the permanent dentition (Andreasen *et al.* 1995a,b, Flores *et al.* 2007, Soares Ade *et al.* 2008). The prognosis of the tooth depends on the measures taken at the time of the accident and/or the period immediately after avulsion. Although immediate replantation is the treatment of choice (Flores *et al.* 2007), clinical experience has shown that most avulsed teeth are replanted only after an extended extra-alveolar time and when dry or stored in inadequately moist conditions (Andreasen *et al.* 1995a,b, Flores *et al.* 2007, Soares Ade *et al.* 2008). Depending on the extra-alveolar time and the storage/transport medium, pulp necrosis and degeneration of the cemental periodontal ligament (PDL) cells may occur. This may lead to inflammatory root resorption, which is the major cause of tooth loss (Hammarström *et al.* 1986, Andreasen *et al.* 1995a,b, Chamorro *et al.* 2008, Panzarini *et al.* 2008, Soares Ade *et al.* 2008).

Successful tooth replantation with a favourable prognosis depends essentially on the maintenance of PDL vitality (Ashkenazi *et al.* 2000, Pileggi *et al.* 2002, Sigalas *et al.* 2004, Chamorro *et al.* 2008, Panzarini *et al.* 2008). Several interim transport media for avulsed teeth have been investigated with respect to their capacity to preserve the vitality of PDL components and dental pulp tissue until the moment of

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replantation (Ashkenazi *et al.* 2000, Pileggi *et al.* 2002, Sigalas *et al.* 2004, Chamorro *et al.* 2008, Gopikrishna *et al.* 2008a,b, Panzarini *et al.* 2008). The ideal storage medium should be able to preserve cell vitality, adherence and clonogenic capacity (Ashkenazi *et al.* 2000) and should be readily available at the site of accident or be easily accessible (Huang *et al.* 1996).

Coconut water is biologically pure, sterile and rich in amino acids, proteins, vitamins and minerals (Campbell-Falck *et al.* 2000). As it is readily accepted by the body, coconut water has been widely consumed to replace fluids, electrolytes (potassium, calcium and magnesium), and sugars lost from the body during heavy physical exercise (Campbell-Falck *et al.* 2000, Gopikrishna *et al.* 2008a,b). This natural isotonic fluid is available in its natural form directly from the coconut or in long-shelf-life packages and plastic bottles mainly in tropical countries, but it has also become increasingly more available in several geographical locations worldwide (Gopikrishna *et al.* 2008a,b). For this reason, its use as a storage medium for avulsed teeth has been investigated recently (Gopikrishna *et al.* 2008a,b). Therefore, the purpose of this study was to evaluate *in vitro* the effectiveness a new storage medium for avulsed teeth, coconut water, in maintaining the viability of human fibroblasts at different times, by the analysis of cell metabolism using the neutral red assay.

Material and methods

Immortalised human fibroblasts of the McConey cell line (American Type Tissue Culture – ATTC, Rockville, MD, USA), originating from knee joint synovial fluid, were cultured in Eagle's minimal essential medium (EMEM; Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 7.5% foetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) in a humidified incubator with 5% CO_2 and 95% air at 37 °C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The cells were subcultured every 3 days until an adequate number of cells were obtained.

Cell viability in the following storage media was evaluated: long-shelf-life coconut water (Socôco, Maceió, AL, Brazil), coconut water with sodium bicarbonate (neutral pH), long-shelf-life whole milk (UHT – ultra high temperature – Companhia Leco de Produtos Alimenticios, Araraquara, SP, Brazil), saline and still mineral water (São Pedro, Manaus, AM, Brazil). Cells cultured in EMEM and pure storage media served as positive and negative controls respectively. The pH of all solutions was measured with a digital pH meter (B374 – Micronal) at room temperature. The pH of the coconut water was adjusted by adding of sodium bicarbonate until a neutral pH of 7.0 was obtained. The milk was maintained at room temperature until use in the cell culture.

Cell viability was determined by the neutral red assay (Borenfreund & Puerner 1985). Initially, the cell culture was trypsinised and 100 μL of EMEM containing approximately 10^4 cells mL^{-1}, counted in a Neubauer chamber (Boeco, Hamburg, Germany), were pipetted and placed in the wells of 96-well plates. All procedures were undertaken in a vertical laminar flow hood. The plates were incubated overnight with 5% CO_2 and 95% air at 37 °C until reaching a minimum of 70% of cell confluence, as observed in an inverted microscope (Olympus CK2; Olympus Optical Co. Ltd., Tokyo, Japan). Then, EMEM was replaced by equal amounts of the storage media and the plates were incubated with 5% CO_2 and 95% air at 37 °C for 1, 2 and 4 h. All tests were performed in triplicate. After the predetermined periods, the plates were prepared for the neutral red assay.

The 0.4% w/v neutral red stock solution (3-amino-m-dimethylamino-2-methylphenazine hydrochloride) in PBS, stored at 4 °C, was diluted in EMEM without CFS until reaching a final concentration of 50 μg mL^{-1} and was incubated overnight at 37 °C. After centrifugation at 1500 rpm for 10 min, 200 μL of this solution was added to each well containing the fibroblasts in contact with the tested storage media for the different times. The cells were incubated with 5% CO_2 and 95% air at 37 °C for 3 h. The culture medium containing the neutral red dye was eliminated and the wells were rinsed with PBS buffer solution. After removal of the PBS buffer, a mixture of 200 μL of acetic acid and ethanol (1 : 50) was added to each well. The plates were undisturbed for 10 min for extraction of the dye. All procedures were undertaken in the vertical laminar flow hood. The colouration produced in the assay was measured by spectrophotometry with an ELISA plate reader (ELX 800; Biotek Instruments Inc., Winooski, VM, USA) at 540 nm wavelength.

The percentage of viable cells after treatments was calculated to determine the effectiveness of the storage media in maintaining cell viability. Data were analysed statistically by ANOVA and the least significant difference (LSD) test at 5% significance level.

Results

The pHs of the solutions were: coconut water = 4.1, saline = 6.4, mineral water = 5.8 and milk = 6.8. The storage media influenced significantly cell viability

($P < 0.05$). Milk had the greatest capacity to maintain cell viability and was significantly better than all other groups ($P < 0.05$). Saline and coconut water with sodium bicarbonate were similar to each other and were significantly better ($P < 0.05$) than coconut water and mineral water. Coconut water had a significantly lower capacity to maintain cell viability compared to milk, coconut water with sodium bicarbonate and saline ($P < 0.05$). The smallest number of viable human fibroblasts was observed after cell exposure to mineral water ($P < 0.05$) (Table 1).

Discussion

Better healing is expected when the replantation of an avulsed tooth is performed immediately after trauma (Andreasen *et al.* 1995a,b, Soares Ade *et al.* 2008). Nevertheless, due to a number of conditions at the moment of the accident, immediate replantation rarely occurs. In these cases, the maintenance of PDL vitality until dental care can be rendered is important for a good prognosis. Two essential factors for the survival of replanted teeth are the interim transport medium and the time elapsed between avulsion and replantation (Söder *et al.* 1977, BlomLöf 1981, BlomLöf *et al.* 1981a, Lindskog *et al.* 1983, Hammarström *et al.* 1986, Andreasen *et al.* 1995a,b, Panzarini *et al.* 2008, Soares Ade *et al.* 2008). Dry storage presents remarkably worse results compared to moist storage and a longer extra-alveolar period is also less favourable to the repair. However, the capacity of the storage medium to preserve cell vitality is considered more critical to the prevention of inflammatory and replacement root resorption than the extra-alveolar time (Söder *et al.* 1977, BlomLöf *et al.* 1981a, Lindskog & BlomLöf 1982, Hammarström *et al.* 1986, Panzarini *et al.* 2008).

Different substances have been evaluated as storage media for exarticulated teeth and the use of coconut water has been investigated more recently. (Lindskog *et al.* 1983, Huang *et al.* 1996, Ashkenazi *et al.* 2000, Pileggi *et al.* 2002, Gopikrishna *et al.* 2008a,b). Each storage medium has characteristics of pH, osmolality and composition that influence directly its capacity to maintain cells viable after a certain time (Lindskog & BlomLöf 1982). Regarding osmolality and pH, the closer to physiological conditions (300 mOsm kg^{-1} and pH 7.0 respectively), the greater the capacity of the medium to preserve cell vitality.

Coconut water is a natural product, which has high osmolality. Because of its composition and ready acceptance by the human body, coconut water has recently been investigated as a potential interim storage/transport medium for exarticulated teeth (Gopikrishna *et al.* 2008a,b). However, coconut water has an acid pH of 4.1, which is deleterious to cell metabolism. In the present study, coconut water was less effective as a storage medium than coconut water with sodium bicarbonate, which indicates that the pH is an important factor to be considered for preservation of cell vitality. During neutralization of the pH, the osmolality of the coconut water increased, and this might also have contributed to maintain the viablility of the fibroblasts. Coconut water with sodium bicarbonate had similar capacity to maintain cell viability as that of saline. However, its indication as a storage medium for avulsed teeth is not feasible under clinical conditions because of the difficulty in neutralizing the coconut water to obtain a pH 7.0.

Gopikrishna *et al.* (2008a,b) have recently reported that coconut water had a greater capacity to maintain cell viability when compared to propolis, HBSS and milk. Those authors used the trypan blue exclusion technique, which determines if the cells are vital or nonvital based on the analysis of cell plasma membrane integrity. However, the sensitivity of this method is low because it does not characterize the metabolic condition of the nonstained cells, which means that the plasma membrane may remain intact, without the cell having any metabolic activity (Tatnall *et al.* 1990).

Milk has still been considered as the best transport medium for storage of exarticulated teeth. In addition to presenting osmolality close to physiological conditions (251–298 mOsm kg^{-1}) (BlomLöf *et al.* 1981b, Rozenfarb *et al.* 1997), milk has good physiological characteristics, preserves the vitality of PDL cells, favours the repair process, and is easily obtained (BlomLöf 1981, BlomLöf *et al.* 1981a,b, Lindskog & BlomLöf 1982, Lindskog *et al.* 1983, Huang *et al.* 1996, Rozenfarb *et al.* 1997, Pileggi *et al.* 2002,

Table 1 Percentage (%) of viable cells after 4 h exposure of the human fibroblasts to each storage medium

Storage Media	Mean (%)
Mineral water	7.1a
Coconut water	14.4b
Saline	55.2c
Coconut water with sodium bicarbonate	50.3c
Milk	80.1d

Different letters indicate statistically significant difference (LSD, $\alpha = 0.05$).

Sigalas *et al.* 2004, Chamorro *et al.* 2008). In the present study, milk was the best storage medium regardless of the exposure time, maintaining fibroblast viability around 80.1% after 4 h of storage.

Conclusion

The capacity of the storage media in maintaining human fibroblast cell viability in a decreasing order was: milk > saline and coconut water with sodium bicarbonate > coconut water > still mineral water.

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