Dental Traumatology

Dental Traumatology 2013; **29**: 319–322; doi: 10.1111/j.1600-9657.2012.01175.x

A multiparametric assay to compare the cytotoxicity of soy milk with different storage media

Emmanuel J. N. L. Silva[1], Carolina B. Rollemberg[2], Tauby de Souza Coutinho-Filho[2], Alexandre A. Zaia[1]

[1]Endodontic Division, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil; [2]Endodontic Division, Department of Restorative Dentistry, State University of Rio de Janeiro, Rio de Janeiro, Brazil

Key words: cell viability; fibroblasts; soy milk; tooth avulsion

Correspondence to: Emmanuel João Nogueira Leal da Silva, Piracicaba Dental School – State University of Campinas-UNICAMP, Endodontic Division, Department of Restorative Dentistry, Av. Limeira 901. – Bairro Areiao, Piracicaba, São Paulo, Brazil
CEP 13414-903
Tel.: (55) 19 2106 5342
Fax: (55) 19 2106 5218
e-mail: nogueiraemmanuel@hotmail.com

Accepted 10 July, 2012

Abstract – *Background/Aim*: The aim of this study was to evaluate the cytotoxicity of soy milk compared with several other storage media [coconut water, Hank′s Balanced Salt Solution (HBSS) and whole milk], assessed through a multiparametric analysis employing 3T3 cells. *Materials and methods*: Plates containing confluent 3T3 fibroblasts were exposed to the various media for 24 h, at 37°C with 5% CO_2, and cell viability was evaluated by a multiparametric assay assessing sequentially, on the same cells, mitochondrial activity (XTT), membrane integrity (neutral red test) and total cell density (crystal violet dye exclusion test). Results from each test were compared by two-way analysis of variance (ANOVA). *Results*: Statistical analysis showed that whole milk, HBSS and soy milk were the most effective media in maintaining cell viability at all tested times ($P < 0.05$). The least amount of viable cells was observed when using coconut water. *Conclusions*: This study shows that the efficacy of soy milk in maintaining the viability of 3T3 fibroblasts is similar to that of HBSS and milk, as shown by three different cell viability tests.

Tooth avulsion is one of the main issues in dental traumatology because it is a severe dental injury. Because of the complexity of this injury, the neurovascular supply is severely compromised in most cases, causing loss of pulp vitality (1). Aetiological factors are trauma after fighting and sports, as well as falls and bumps against hard objects (2). The reported incidence of tooth avulsion is approximately 1–16% of all traumatic injuries to the permanent dentition (3,4).

The prognosis of the tooth depends on the measures taken at the time of the accident and the period immediately after avulsion. Although immediate replantation is the treatment for choice (5), clinical experience has shown that most avulsed teeth are replanted only after an extended extra-alveolar time (3–5). Depending on the extra-alveolar time and the storage/transport medium, pulp necrosis and degeneration of the cementum and periodontal ligament (PDL) cells may occur. This may lead to inflammatory root resorption and replacement resorption, which are the major causes of tooth loss (2,3,6,7). It becomes necessary to choose a suitable storage medium for maintaining the viability of periodontal ligament cells, avoiding further damage to the tooth.

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 replantation (1,6–10). The ideal storage medium should preserve cell vitality, adherence and clonogenic capacity (11) and should be readily available at the site of accident (12). To date, several types of media have been used for the storage of avulsed teeth. Some examples are saliva, milk, Hank's Balanced Salt Solution (HBSS), Save-A-Tooth system (Phoenix-Lazerus, Shartlesville, PA, USA) and ViaSpan (DuPont Phamaceuticals, Wilmington, DE, USA). Other storage media including egg white, powdered milk, Gatorade (The Gatorade Co., Chicago, IL, USA) and propolis have been recently studied and tested (1,6–14).

Soy milk, the water extract of soybean, contains no cholesterol or lactose and very small amounts of saturated fatty acid (15,16). In addition, it may play a role in the prevention of chronic diseases such as atherosclerosis, cancer, osteoporosis and menopausal disorders

(17), as well as being an excellent culture medium for cell growth and biochemical activities (18). A recent study showed that soy milk in contact with periodontal ligament cells promoted good cell viability and is recommended as a good storage media (8).

The simultaneous evaluation of different cell viability parameters may more accurately identify any possible cytotoxic effects of storage media with immortalized cells intimately related to the *in vivo* tissue (19). Therefore, the aim of this study was to compare the cytocompatibility of four storage media, namely long-shelf-life coconut water (Taeq, Fortaleza, CE, Brazil), long-shelf-life whole milk (Parmalat, São Paulo, SP, Brazil), long-shelf-life soy milk (Ades, Belo Horizonte, MG, Brazil) and Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY, USA). Cytocompatibility was assessed by a multiparametric assay employing 3T3 fibroblasts.

Materials and methods

Cell viability in the following storage media was evaluated: long-shelf-life coconut water (Taeq), long-shelf-life whole milk (Parmalat), long-shelf-life soy milk (Ades) and Hank's Balanced Salt Solution (Gibco BRL). Cells cultured in DMEM served as a positive control. The pH of all solutions was measured with a digital pH meter (QM-400; Quimis, São Paulo, SP, Brazil) at room temperature. The osmolality was tested with an automatic cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany).

Balb C 3T3 cells (American Tissue Type Collection, ATCC, Manassas, VA, USA) were cultured in Dulbecco modified Eagle medium (DMEM) (Gibco) supplemented with 10% foetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA), 100 μg ml^{-1} of streptomycin and 100 mg ml^{-1} of penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) for 5 minutes, and aliquots of separated cells were subcultured for 24 h at 37°C on 96-well culture plates at an initial cell density of 10 000 cells per well and subsequently exposed to the different storage media for 24 h. All storage media were tested on three replicates and three different assays.

After 24 h of cell exposure to each storage media, cytotoxicity was evaluated with a commercial kit (Cytotox, Xenometrix, Germany) that evaluates three different cell viability parameters sequentially on the same cell culture (19,20): XTT, neutral red (NR) and crystal violet dye elution (CVDE). The XTT test is based on the ability of mitochondrial enzymes from metabolically active cells to reduce 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) molecules to a soluble salt of formazan, detectable by its absorbance at 480 nm, as measured by a spectrophotometer (Urit 660, Urit, China). The same cells submitted to the XTT test were washed and assayed with the neutral red uptake test (NR), which determines the levels of viable cells through their membrane integrity. The vital dye NR is incorporated through endocytosis and accumulates preferentially on the lysosomes of membrane intact viable cells. After 3 h of exposure to the dye, cells were fixed and the NR was extracted and measured by the optical density (OD) of the supernatant at 540 nm, which directly relates to the proportion of viable cells. After the NR test, fixed cells were washed and evaluated for the total density of cells adhered, as estimated by the crystal violet dye exclusion test (CVDE). CVDE is a simple assay that evaluates cell density by staining DNA; after elimination of excess dye, the absorbance at 540 nm is proportional to the amount of cells in the well.

Two-way Analysis of Variance (ANOVA) was employed to test the interactions of two sources of variation (storage media and test method) with the proportions of viable cells for each storage media, as compared to the control group. Follow-up comparison between the groups was made using Tukey multiple comparison test (at 95% confidence interval level, $a = 0.05$). All statistical analyses were performed with SPSS 10 (SPSS, Inc., Chicago, IL, USA).

Results

Figure 1 shows cell viability as evaluated by three different assays, after exposure to 24-h of the different storage media, expressed as a percentage of the control (cells exposed to DMEM). As seen in panels A, B and C, only coconut water has cytotoxic effects, as measured by all three methods employed. No significant difference was found between soy milk, whole milk and HBSS ($P < 0.05$). No statistical difference was found between the different test methods ($P < 0.05$). The results of pH and osmolality are expressed in Table 1.

Discussion

In this study, the cytotoxicity of four available storage media were tested, employing an *in vitro* methodological strategy that differs from most previous works on these materials by employing a multiparametric assay with three different cell viability tests. In this manner, three different parameters were evaluated on the same samples: (1) mitochondrial metabolism and respiratory toxicity, (2) lysosomal integrity and membrane permeability and (3) the presence of DNA and cell proliferation. This method increases the chance of detection of cytotoxic effects, allows correlation of different parameters, and sometimes provides hints about the mechanisms of toxicity (19,20). A 3T3 fibroblast cell line was used in this study for easy preparation and handling. Fibroblasts are major constituents of connective tissue and the predominant cell type of periodontal ligament. In addition, studies with established cell lines are used because of the reproducibility of the results; furthermore, they multiply rapidly and have an unlimited lifespan (21–23).

Milk, HBSS, coconut water and most recently soy milk have been used as storage media. HBSS is a standard saline solution that is widely used in biomedical research to support the growth of many cell types and is recommended by the International Association of

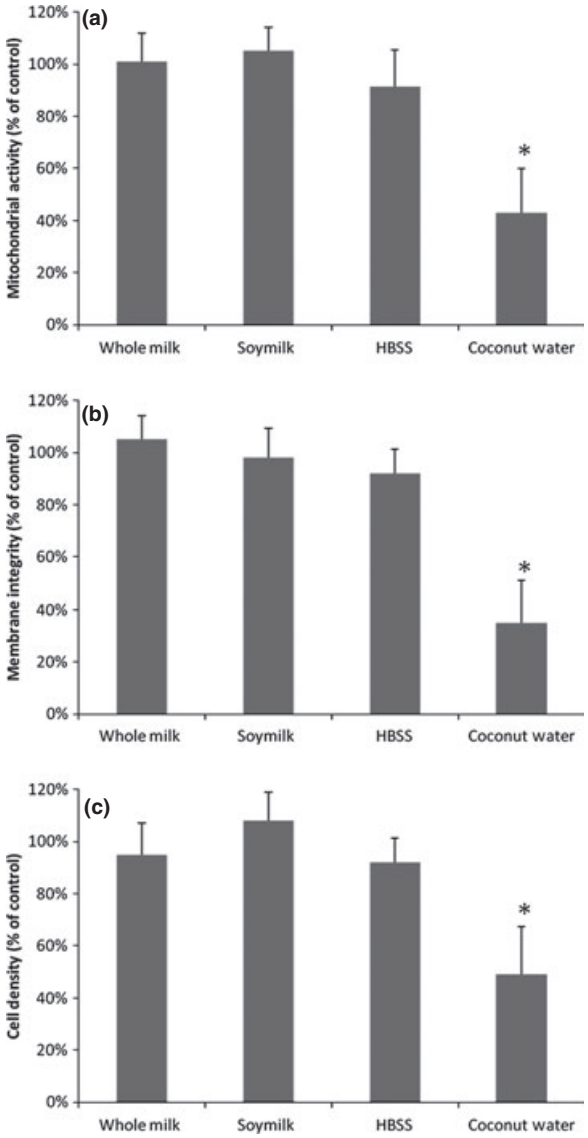

Fig. 1. Cytotoxic effects of storage media on 3T3 cells by XTT (a), neutral red (b) and crystal violet tests (c), expressed as percentage of control (cells exposed to culture medium). Bars indicate mean ± SD. (*) mean statistically significant differences between tested groups ($P < 0.05$) and in the same assay.

Dental Traumatology as a storage medium for avulsed teeth (5,9). The osmolality and pH of HBSS are 270–290 mosmol kg^{-1} and 7.2, respectively. It is nontoxic and pH balanced and contains many essential nutrients (5,6,8,9). HBSS has been commercially available as Save-A-Tooth (Save-A-Tooth Inc., Pottstown, PA, USA) as a storage medium for avulsed teeth, although it is not yet widely available in pharmacies or drugstores around the world. Milk is regarded as a convenient storage medium for an avulsed tooth because it is easy to get in the event of an accident and it can maintain PDL cells. In addition, milk has a physiologically compatible pH and osmolality, many essential nutrients and growth factors (6,12,14). Soy milk is an aqueous solution rich in protein, amino acids, vitamins and minerals essentials for cell nutrition and maintenance. It also has a physiological pH and is gradually becoming available to a wider population (15,16). Several studies have shown the excellent biological properties of soy milk, reporting the excellent potential of soy milk as a storage medium and proliferator of several cell types (17,18,24,25).

Table 1. pH and osmolality of different storage medium

Material	Osmolality (mOsmol kg^{-1})	pH
Coconut water	370	4.5
Whole milk	284	6.8
Soymilk	261	7.2
DMEN	313	8.1
HBSS	283	7.6

HBSS, Hank's Balanced Salt Solution.

Our results indicated that HBSS, milk and soy milk were effective in maintaining 3T3 cell viability because they could preserve viability at over 90%. The result of HBSS' and milk's effectiveness is in agreement with other studies (5,6,8,9,12,14). The results of this study also showed that soy milk is as effective a storage medium for avulsed teeth as HBSS and milk. One reason for these results can be the excellent biological properties of soy milk. In this study, soy milk showed a physiologically compatible pH and osmolality. Both physiological osmolality and pH are important factors in preserving the viability of PDL cells. It has been reported that the growth of cells mainly occurs at an osmolality of 230–400 mosmol kg^{-1} and a pH of 6.6 to 7.8. HBSS and milk also exhibit physiological pH and osmolality.

Similar cytotoxic results were found in the Moazami study (8), which showed that soy milk can be an excellent storage medium for avulsed teeth. In the previous study, cytotoxicity was observed using a Trypan blue assay. This assay is less sensitive than the multiparametric assay, as it does not characterize the true metabolic condition of cells not stained with Trypan blue. This means that the cell membrane is intact, although the cell may not have any kind of metabolic activity (24). This study further supports the results presented above, showing cytotoxicity values for soy milk that are similar to the widespread storage medium.

According to the results of this study, it could be concluded that soy milk can maintain cell viability at levels similar to solutions considered 'gold-standards' for avulsed teeth such as whole milk and HBSS, supporting its use in cases of dental trauma.

References

1. Martin MP, Pileggi R. A quantitative analysis of Propolis: a promising new storage media following avulsion. Dent Traumatol 2004;20:85–9.
2. Trope M. Avulsion of permanent teeth: theory to practice. Dent Traumatol 2011;27:281–94.
3. Soares AJ, Gomes BP, Zaia AA, Ferraz CC, de Souza-Filho FJ. Relationship between clinical-radiographic evaluation and outcome of teeth replantation. Dent Traumatol 2008;24:183–8.
4. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors 1. Diagnosis of healing complications. Endod Dent Traumat 1995;11:51–8.

5. Flores MT, Andersson L, Andreasen JO, Bakland LK, Malmgren B, Barnett F et al. International Association of Dental Traumatology. Guidelines for the management of traumatic dental injuries. II. Avulsion of permanent teeth. Dent Traumatol 2007;23:130–6.
6. Chamorro MM, Regan JD, Opperman LA, Kramer PR. Effect of storage media on human periodontal ligament cell apoptosis. Dent Traumatol 2008;24:11–6.
7. Gjertsen AW, Stothz KA, Neiva KG, Pileggi R. Effect of propolis on proliferation and apoptosis of periodontal ligament fibroblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:843–8.
8. Moazami F, Mirhadi H, Geramizadeh B, Sahebi S. Comparison of soymilk, powdered milk, Hank′s balanced salt solution and tap water on periodontal ligament cell survival. Dent Traumatol 2012;28:132–5.
9. Gopikrishna V, Baweja PS, Venkateshbabu N, Thomas T, Kandaswamy D. Comparison of coconut water, propolis, HBSS, and milk on PDL cell survival. J Endod 2008;34:587–9.
10. Hwang JY, Choi SC, Park JH, Kang SW. The use of green tea extract as a storage medium for the avulsed tooth. J Endod 2011;37:962–7.
11. Ashkenazi M, Marouni M, Sarnat H. In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in four media at room temperature. Endod Dent Traumatol 2000;16:63–70.
12. Huang SC, Remeikis NA, Daniel JC. Effects of long-term exposure of human periodontal ligament cells milk and other solutions. J Endod 1996;22:30–3.
13. Khademi AA, Saei S, Mohajeri MR et al. A new storage medium for an avulsed tooth. J Contemp Dent Pract 2008;9:25–32.
14. dos Santos CL, Sonoda CK, Poi WR, Panzarini SR, Sundefeld ML, Negri MR. Delayed replantation of rat teeth after use of reconstituted powdered milk as a storage medium. Dent Traumatol 2009;25:51–7.
15. Ng KH, Lye HS, Easa AM, Liong MT. Growth characteristics and bioactivity of probiotics in tofubased medium during storage. Annals Microbiol 2008;58:477–87.
16. Wang YC, Yu RC, Chou CC. Growth and survival of bifidobacteria and lactic acid bacteria during the fermentation and storage of cultured milk drinks. Food Microbiol 2002;19:501–8.
17. Liu JR, Chen MJ, Lin CW. Characterization of polysaccharide and volatile compounds produced by kefir grains in soymilk. J Food Sci 2002;67:104–8.
18. Scalabrini P, Rossi M, Spettoli P, Matteuzzi D. Characterization of Bifidobacterium strains for use in soymilk fermentation. Int J Food Microbiol 1998;39:213–9.
19. Scelza MZ, Linhares AB, da Silva LE, Granjeiro JM, Alves GG. A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. Int Endod J 2012;45:12–8.
20. De-Deus G, Canabarro A, Alves G, Linhares A, Senne MI, Granjeiro JM. Optimal citocompatibility of a bioceramic nanoparticulate cement in primary human mesenchymal cells. J Endod 2009;35:1387–90.
21. Bouillaguet S, Wataha JC, Tay FR, Brackett MG, Lockwood PE. Initial in vitro biological response to contemporary endodontic sealers. J Endod 2006;32:989–92.
22. Loushine BA, Bryan TE, Looney SW, Gillen BM, Loushine RJ, Weller RN et al. Setting properties and cytotoxicity evaluation of a premixed bioceramic root canal sealer. J Endod 2011;37:673–7.
23. Silva E, Accorsi-Mendonça T, Almeida J, Ferraz C, Gomes B, Zaia A. Evaluation of cytotoxicity and up-regulation of gelatinases in human fibroblast cells by four root canal sealers. Int Endod J 2011;45:49–56.
24. Onuegbu AJ, Olisekodiaka JM, Onibon MO, Adesiyan AA, Igbeneghu CA. Consumption of soymilk lowers atherogenic lipid fraction in healthy individuals. J Med Food 2011;14:257–60.
25. Ewe JA, Abdullah WN, Liong MT. Viability and growth characteristics of lactobacillus in soymilk supplemented with B-vitamins. Int J Food Sci Nutr 2010;61:87–107.