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ORIGINAL ARTICLE

The efficacy of *Salvadora persica* extracts in preserving the viability of human foreskin fibroblasts

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KEYWORDS

Avulsion;
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Abstract *Objective:* To evaluate the efficacy of *Salvadora persica* hexane and ethanol extracts in preserving the viability of human foreskin fibroblasts.

Materials and methods: Normal human foreskin cells were cultivated in Dulbecco modified Minimum Essential Medium (D-MEM) supplemented with 10% fetal bovine serum and 2 mM of L-glutamine. Cell pellets were suspended in the following test solutions: (1) Hank's Balanced Salt Solution (HBSS); (2) homogenized milk; (3) hexane extract of *S. persica*; or (4) ethanol extract of *S. persica*. D-MEM with no serum was used as a positive control. For each condition, cell count was adjusted to 8×10^5 cells/ml, and the cells were incubated in the solutions for either 30, 60, or 120 min. Subsequently, the nonviable cells were separated from the viable cells using the trypan blue dye stain. The ratio of viable to nonviable cells was recorded using a cell counter. Statistical analysis of the data was accomplished by one-way analysis of variance using SPSS Version 16. The level of significance was 5% ($p < .05$).

Results: We did not detect a significant difference when comparing the percentage of viable cells in test solutions at the three incubation periods (30 min, $p = 0.478$; 60 min, $p = 0.606$; 120 min, $p = 0.091$). Homogenized milk preserved the viability of foreskin fibroblasts better than all other

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tested solutions. Incubation of cells in *S. persica* hexane and ethanol extracts resulted in a similar percentage of viable cells to incubation of cells in HBSS for each incubation period.

Conclusions: *S. persica* hexane and ethanol extracts should be considered an alternative storage medium to HBSS.

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1. Introduction

Dental injuries, including tooth avulsion, are the most common of all facial injuries. Tooth avulsion occurs in 1–16% of all dental injuries (Andersson et al., 2012; Soares Ade et al., 2008). The prognosis of the avulsed tooth depends on the time of the injury and the extra-alveolar time (Andersson et al., 2012). In cases where these factors are unfavorable, pulp necrosis and degeneration of periodontal ligament (PDL) cells may occur, which leads to inflammatory root resorption and eventually tooth loss (Soares Ade et al., 2008). In fact, maintaining PDL cell viability promotes a successful tooth replantation (Sigalas et al., 2004). Tooth replantation is the treatment of choice for tooth avulsion, however, replantation cannot always be carried out immediately (Andersson et al., 2012). In these cases, the avulsed tooth must be stored until tooth replantation can occur. Typically, studies use either PDL cells (Blomlof, 1981; Gopikrishna et al., 2008; Hiltz and Trope, 1991; Moreira-Neto et al., 2009; Ozan et al., 2008; Rajendran et al., 2011) or human foreskin fibroblasts (Courts et al., 1983; Rozenfarb et al., 1997) to evaluate the efficacy of different storage media solutions for an avulsed tooth.

Salvadora persica (*S. persica*; miswak) is a member of the Salvadoraceae family, and among the 182 plant species used to prepare brushing sticks, *S. persica* is the most commonly used (Elvin-Lewis, 1982). Several studies have shown the antimicrobial effects of *S. persica* against several oral pathogens, and the ability of *S. persica* to prevent dental caries and reduce gingivitis and dental plaque (Al-Otaibi et al., 2003; Ezoddini-Ardakani, 2010). Thus far only a few studies have evaluated the cytotoxicity of *S. persica* extracts (Al-Samh and Al-Nazhan, 1997; Balto et al., 2014). However, the efficacy of *S. persica* extract in preserving human fibroblast cell viability is unknown. Accordingly, we compared the efficacy of *S. persica* hexane and ethanol extracts to preserve the viability of human foreskin fibroblasts *in vitro* with Hank's Balanced Salt Solution (HBSS), homogenized milk, and tissue culture medium [Dulbecco modified Minimum Essential Medium (D-MEM) with no serum].

2. Materials and methods

2.1. Preparation of ***S. persica*** extracts

The study was registered and conducted as per the ethical guidelines of the College of Dentistry Research Centre (CDRC Registration Number FR0027). The roots of *S. persica* were collected from Almukhwah (near Al-Baha area of Saudi Arabia), and a taxonomist identified the plant (Balto et al., 2014). A voucher specimen (#1745) was deposited at the Herbarium Centre, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for future reference. The hexane extract was prepared by percolating 100 g of dried *S. persica* root powder in hexane for 72 h, adding fresh solvent every 24 h. The ethanol extract was prepared similarly, except using 90% ethanol (absolute ethanol containing 10% water). Extracts were freeze-dried to completely remove the solvents. Stock preparations of *S. persica* extracts were made in dimethyl sulfoxide (DMSO) at 100 mg/ml and were frozen at −30 °C. The working dilutions of *S. persica* extracts were made in physiological saline at a pH of 7.4.

2.2. Experimental procedures

The third passage of normal human foreskin cells was obtained from the Stem Cell Unit at the College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia. Cells were cultivated in D-MEM (Invitrogen, USA) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The media also contained penicillin (100 IU) and streptomycin (100 μg/ml). Cells in tissue culture flasks (TPP, Switzerland) were incubated at 37 °C in an environment of 5% carbon dioxide and 75% relative humidity. Cell monolayers were washed with phosphate buffered saline depleted of calcium and magnesium ions and digestion was carried out with 0.25% trypsin solution (Invitrogen, USA). Cells were collected in complete cell culture medium and pelleted by centrifugation at 1000×*g* for 10 min. The cell count was adjusted to 8×10^5 cells/ml in each tube and resuspended in the following test solutions: (1) HBSS; (2) homogenized milk; (3) 2.5 mg/ml hexane extract of *S. persica*; or (4) 2.5 mg/ml ethanol extract of *S. persica*. Tissue culture medium (D-MEM with no serum) was used as a positive control. Tubes were incubated in a general incubator (JSGI-150T, JS Research Inc., South Korea) at 37 °C, 5% carbon dioxide, and 75% relative humidity for an incubation period of 30, 60, or 120 min. The number of viable cells was measured using the trypan blue dye exclusion test (Phillips, 1973). The total viable and non-viable cell count was carried out using a cell counter (Invitrogen, USA).

2.3. Statistical evaluation

Statistical analysis of the data was accomplished by one-way analysis of variance using SPSS Version 16. The level of significance was 5% ($p < 0.05$).

3. Results

There was no significant decrease in the number of viable human foreskin fibroblasts after storage in any of the tested solutions for any of the experimental time periods compared to the control (Table 1). However, storage in homogenized milk resulted in a higher average percentage of viable cells at all experimental time points compared

Table 1 Percentage of viable human foreskin fibroblasts (average ± SD) in test solutions at 30, 60, and 120 min.

Groups	30 min	60 min	120 min
Tissue culture medium[a]	85.7 ± 2.31	87 ± 1.0	77.3 ± 1.15
Hank's Balanced Salt Solution	83 ± 1.0	76.3 ± 0.58	60.7 ± 1.53
Homogenized milk	90.7 ± 0.58	90.0 ± 0.0	89.3 ± 2.08
S. persica hexane extract (2.5 mg/ml)	81.3 ± 1.5	80.7 ± 0.58	62.7 ± 0.58
S. persica ethanol extract (2.5 mg/ml)	82.3 ± 0.58	79.7 ± 0.58	66 ± 6.08

[a] D-MEM with no serum.

to those stored in all other test solutions. We did not observe a significant difference in the average percentage of viable cells in test solutions between the three incubation periods (30 min, $p = .478$; 60 min, $p = .606$; and 120 min, $p = .091$). The percentage of viable cells was only slightly reduced after storage in both hexane and ethanol extract of *S. persica* for 30 min in comparison with the control, however, differences increased after longer incubation periods.

4. Discussion

The outcome of avulsed tooth replantation after dry storage and a long extra-alveolar time is significantly worse compared to moist storage and a short extra-alveolar time. In order to prevent inflammation and root resorption, the ability of the storage medium to preserve the viability of PDL cells is more important than extra-alveolar time (Moreira-Neto et al., 2009). Because of the importance of PDL cell viability prior to replantation and because the length of survival of a replanted tooth correlates with the amount of periodontal membrane (Hammer, 1955), several types of media have been tested for their ability to preserve PDL fibroblast viability. Thus far, no study has evaluated the effectiveness of *S. persica* extracts as a storage medium for avulsed teeth.

Fibroblast-like cells predominate in human PDL cell culture. Several metabolic and morphologic similarities exist between PDL fibroblasts and human skin fibroblasts *in vitro* (Blomlof, 1981), which make human skin fibroblasts an ideal cell line to test PDL fibroblast storage media. To enumerate viable cells, studies have used either collagenase – dispase enzymes (Martin and Pileggi, 2004; Gopikrishna et al., 2008) or trypan blue exclusion staining (Sigalas et al., 2004; Ozan et al., 2008). Since trypan blue exclusion staining easily and quickly differentiates nonviable from viable cells (Ozan et al., 2008), we chose this technique for our study. On the other hand, the use of human foreskin fibroblasts presents some limitation to our study which should be considered when interpreting the results. Previous studies have recommended using extracted teeth, as a simulation of avulsion (Martin and Pileggi, 2004; Haas et al., 2008). However, trauma induced by different clinicians during extractions, prior to obtaining PDL fibroblasts, could translate to variability of PDL cell vitality counts (Rajendran et al., 2011). Therefore, there are both advantages and disadvantages to using either skin fibroblasts or PDL fibroblasts for *in vitro* studies. A second limitation of our study was using trypan blue staining to assess cell viability because this technique assesses neither the physiologic health nor the metabolic capabilities of the cells. Furthermore, this method cannot distinguish between necrotic and apoptotic cells (Rajendran et al., 2011). Finally, because homogenized milk is the most efficient and easily available storage medium for avulsed teeth, our study should be considered as the initial step for future investigations of the various clinical applications of *S. persica*.

HBSS is a standard saline solution that supports the growth of many cell types and is used extensively in biomedical research (Krasner and Person, 1992). In our study, incubation of human skin fibroblasts in HBSS for either 60 or 120 min resulted in the lowest percentage of viable cells compared to any other experimental solution. This result contradicts other studies, which report that cell viability in HBSS is better than milk even after 12 h (Hiltz and Trope, 1991; Huang et al., 1996). These inconsistent outcomes may be a result of the temperature of HBSS or the type of milk used in each study. Milk is widely accepted as an effective storage medium (Sigalas et al., 2004), and its efficacy can last from 3 to 48 h (Ashkenazi et al., 1999). The physiologic osmolality (Elvin-Lewis, 1982) and the nutrients and growth factors in milk (Belford et al., 1995) may contribute to its increased ability to preserve cell viability. In our experiments, incubating human skin fibroblasts in milk resulted in a higher percentage of viable cells than observed in any other experimental solution at all experimental incubation periods. This result concurs with previous studies (Ashkenazi et al., 1999; Sigalas et al., 2004).

In order to determine if there is a threshold exposure to *S. persica* extracts that becomes toxic to the cells, additional *in vitro* and *in vivo* studies should be performed with variable storage times and concentrations of *S. persica* extracts. Additionally, currently there are no available antimicrobial or anti-inflammatory storage media that can also effectively maintain PDL cell viability. Because of the antimicrobial (Al-Otaibi et al., 2003; Ezoddini-Ardakani, 2010) and anti-inflammatory properties (Ahmad et al., 2011; Ibrahim et al., 2011) of *S. persica*, future research may establish that these properties improve the prognosis of avulsed teeth by preventing resorption that often leads to the loss of replanted teeth.

5. Conclusions

We conclude that homogenized milk preserves the viability of human foreskin fibroblasts better than HBSS, DMEM, and *S. persica* hexane and ethanol extracts. Because storage in *S. persica* hexane and ethanol extracts resulted in a similar percentage of viable cells as storage in HBSS, these extracts should be considered an alternative storage media to HBSS.

Conflict of interest

The authors of this study confirm that there are no known conflicts of interest associated with this publication.

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