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Influence of Storage Media Containing *Salvia officinalis* on Survival of Periodontal Ligament Cells

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Abstract

Aim: The purpose of this study was to determine the ability of *Salvia officinalis* (*S. officinalis*) extracts to serve as a storage medium for the maintenance of periodontal ligament (PDL) cell viability of avulsed teeth.

Methods and Materials: PDL cells were obtained from healthy third molars and cultured in Dulbecco's Modified Eagle's Medium (DMEM). Cultures were subjected to 4, 2.5, 1.5, and 0.5% *S. officinalis* solutions, Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), and tap water. Tissue culture plates were incubated with experimental media at 37ºC for 1, 3, 6, 12 or 24 hours. PDL cell viability was assessed by trypan blue exclusion. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) complemented by the Tukey's test. The level of significance was 5% ($p < 0.05$).

Results: The results showed 2.5% *S. officinalis* was a more effective storage medium than the other experimental solutions ($p<0.05$). Only at 1 hour and 3 hours was there found similar effect between 2.5% *S. officinalis* and HBSS. At 24 hours, 2.5% *S. officinalis* was found to be significantly better than the other solutions tested.

Conclusion: *S. officinalis* can be recommended as a suitable transport medium for avulsed teeth.

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Clinical Significance: The findings of this study support the use of *S. officinalis* as another option for clinicians to use to store and transport avulsed teeth until reimplantation procedures can be done.

Keywords: *Salvia officinalis*, avulsed teeth, PDL cells, storage media, cell culture

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Introduction



The reported incidence of complete avulsion of teeth ranges from 1 to 16% of all traumatic injuries to the permanent dentition.[1] When avulsion occurs, the avulsed tooth should be immediately replanted at the site of the accident to prevent further damage to the periodontal ligament (PDL) cells from desiccation. However, immediate repositioning of teeth is not always possible under certain conditions. In such situations a storage medium is used to preserve PDL cell viability. The choice of a suitable storage medium for maintenance of maximum PDL cell survival until replantation is very important. Today, several media have been used for storage of an avulsed tooth such as tap water, saliva, milk, Hank's balanced salt solution (HBSS), ViaSpan, culture medium, and propolis.[2-4]

Salvia, the largest genus of *Lamiaceae*, includes approximately 900 species widespread all over the world. This genus is represented in Turkish flora by 88 species and 93 taxa, 45 of which are endemic.[5] Some of the members of this genus are of economical importance since they have been used as aromatic agents in perfumery and cosmetics. *Salvia officinalis* (*S. officinalis*) (*sage*) extracts have been credited with a long list of medicinal uses such as spasmolytics, antiseptics, and astringents.[6] Some phenolic compounds of plants belonging to this genus have also demonstrated excellent antimicrobial activity as well as scavenging activity of their active oxygen forms such as superoxide anion radicals, hydroxyl radicals, and singlet oxygen.[7] These inhibit lipid peroxidation,[8] and the corresponding extracts have been widely used to stabilize fat and fat containing foods.[9] A survey of the literature found only a small amount of information was available on the antiviral and antitumoral potentials of some *Salvia* species. For example, antiviral properties of *S. officinalis* and *S. fruticosa* have previously been reported[10,11] as well as antitumoral properties, e.g., tanshinones from *S. miltiorrhiza*.[12] For the latter activity, the aforesaid rosmarinic acid could also be taken into consideration. On the other hand, anti-inflammatory activities of some of the *Salvia* species had been reported previously.[13]

This study compared HBSS with a natural product, *S. officinalis*, in terms of the enhancement of PDL cell viability.

Methods and Materials

Preparation of Salvia Extract



Using a Soxhlet apparatus 100 g of dried plant material from *S. officinalis* was extracted with methanol (MeOH) over a 6 hour period which yielded 11.08% w/w of extract.[14] Then the extract was combined with chloroform and water to obtain the polar and non-polar extracts. In this assay the polar extract was used. The extract obtained was lyophilized and kept in the dark at +4ºC until used.

Primary Culture of Human PDL Cells

The study was performed at Cumhuriyet University, School of Dentistry Hospital. The protocol was approved by the Ethics Committee of Cumhuriyet University, School of Medicine. Volunteers were informed, in writing, as to the purpose of the study and procedures involved.

The PDL cells were obtained from clinically healthy third molar teeth extracted for orthodontic purposes. The teeth were extracted as atraumatically as possible and washed in sterile saline solution to eliminate residual blood. PDL tissues were scraped with a #15 scalpel using aseptic techniques and then transferred to the culture medium.

The PDL samples were cut into 1-2 mm^3 pieces and then washed twice with HBSS. Thereafter, the cut biopsies were placed into tissue culture flasks (25 cm^2). The explants were incubated with culture medium consisting of the following:

1. Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA)
2. *10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
3. *Glucose (4.5 g/l), $NaHCO_3$ (3.7 g/L)
4. *Penicillin (100 U/mL)
5. *Streptomycin (100 mg/mL)
6. *Amphotericin (2.5 mg/mL) supplemented with 10% heat inactivated fetal calf serum (FCS) (Pan Systems, Aidenbach, Germany).

*items obtained from Biochrom KG, Berlin, Germany

Cells were grown at 37ºC in a humidified atmosphere of 10% CO_2 in air. The culture medium was renewed twice per week until the cells reached confluency. Sub cultivation cells were detached from the culture flasks with 0.25% Trypsin/EDTA Solution (Sigma) for 3–5 minutes. Cells used for the experiments proliferated in logarithmic phase between the 7^{th} and 12^{th} passages. Cell morphology was examined using phase contrast microscopy (TNM, Nikon, Tokyo, Japan).

Exposure of PDL Cultures to Different Solutions

On the day of treatment, the culture medium was drained from each well and the cells were exposed to 2 mL of the different experimental solutions. The storage solutions used in the experiments were as follows:

1. Phosphate buffered saline (PBS) containing 4% *S. officinalis*
2. PBS containing 2.5% *S. officinalis*
3. PBS containing 1.5% *S. officinalis*
4. PBS containing 0.5% *S. officinalis*
5. HBSS
6. PBS alone
7. Tap water

Assessing Cell Viability Using Trypan Blue Exclusion

Following exposure the medium was removed from the wells after 1, 3, 6, 12, and 24 hours then washed with 2 mL sterile PBS followed by the addition of 100 µL of 0.25% trypsin and incubation of the plates at 37ºC for 5-10 minutes. 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 minutes. Next, a 20 µL aliquot was removed and placed under a cover slip on a hemocytometer and both the viable and the non-viable cells were counted under the microscope. The number of viable cells harvested from each petri dish was obtained by the following mathematical equation:

$$UC \times D \times 10^4 / \#SQ$$

Where UC = unstained cell count (viable cells), D = the dilution of the cell suspension, and #SQ = number of squares of the hemocytometer counted.

The viability percentage of the cell population of each Petri dish was obtained by applying the following mathematical equation:

$$UC/TC \times 100$$

Where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells).

Each experiment was repeated four times.

Statistical Analysis

Statistical analysis of the data was accomplished using a one-way analysis of variance (ANOVA) complemented by the Tukey's test. The level of significance was 5% ($p < 0.05$).

Results

The mean percentage values of cell viability are shown in Table 1.

Table 1. Mean percentage of cell viability.

Medium	1 Hour (SD)	3 Hours (SD)	6 Hours (SD)	12 Hours (SD)	24 Hours (SD)
4% *S. officinalis*	97.00 (1.8)	70.00 (2.9)	50.00 (1.8)	33.00 (2.9)	25.00 (1.8)
2.5% *S. officinalis*	98.00 (1.1)	85.00 (2.5)	65.00 (1.8)	45.00 (1.8)	35.00 (3.6)
1.5% *S. officinalis*	97.00 (1.8)	80.00 (5.4)	45.00 (2.9)	36.00 (2.9)	25.00 (2.5)
0.5% *S. officinalis*	97.00 (1.8)	75.00 (2.9)	40.00 (4.0)	30.00 (1.8)	20.00 (2.9)
HBSS	98.00 (0.8)	80.00 (4.08)	50.00 (1.8)	35.00 (1.8)	25.00 (1.8)
PBS	97.00 (1.8)	70.00 (1.8)	40.00 (1.9)	25.00 (1.8)	12.00 (1.8)
Tap Water	25.00 (1.8)	3.75 (0.9)	1.25 (0.5)	0	0

The efficacy of 2.5% *S. officinalis* at 6, 12, and 24 hours was found significantly better than HBSS ($p<0.05$). At 1 hour and 3 hours, there was no significant difference between 2.5% *S. officinalis* and HBSS. At 3 hours, 4% *S. officinalis* was found to be significantly better than HBSS while at the other time intervals the efficacy of these two solutions were not found to be significantly different from each other. In addition, no significant difference was found between 1.5% *S. officinalis* and HBSS at any of the time intervals. When *S. officinalis* solutions were compared with each other, there was no significant difference at 1 hour. Whereas, at 3, 6, 12 and 24 hours, 2.5% *S. officinalis* was found to be significantly better than the others, except at 3 hours when compared to 1.5% *S. officinalis*. Figure 1 shows time dependent results according to experimental solutions on cell viability.

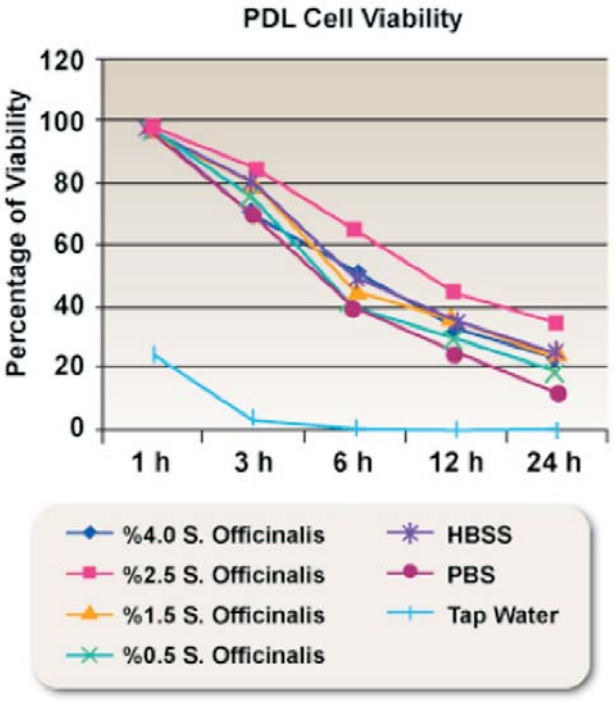


Figure 1. PDL cell viability.

Discussion

Avulsion of the teeth is the worst of the dentoalveolar injuries. Usually avulsion involves a single tooth and the most frequently avulsed tooth is the maxillary central incisor.[15] Treatment of such injuries must be geared toward early reestablishment of PDL cellular physiology.

Successful replantation of avulsed teeth is dependent upon the prevention or limitation of inflammatory and replacement root resorption (ankylosis).[16,17] Clinical studies have shown inflammatory resorption has an early onset, progresses rapidly, and is associated with pulpal infection, contamination of the root surface, and an extraoral drying time of 15 minutes or more.[16,18-21] Inhibition of inflammatory resorption involves removing the necrotic tissue and filling the root canal with calcium hydroxide.[15] Replacement resorption may have a delayed onset and has been correlated with extensive PDL damage, a lengthy extraoral storage time, and prolonged or rigid splinting.[20-23]

Human PDL fibroblasts behave differently in a cell culture than gingival fibroblasts with respect to growth rates and to protein and collagen production.[24] Although lip, gingival, and PDL fibroblasts have similar morphology, they do not behave the same in a culture.[25] Protein and collagen production are significantly greater in PDL cells, and growth rates differ in fibroblast cells obtained from the human PDL which may more accurately reflect the ability of PDL cells on an avulsed tooth to remain viable in a transport medium than fibroblasts obtained from other sources. In recognition of this phenonmenon a human PDL fibroblast cell culture model for evaluation of the efficacy of *S. officinalis* extract solutions in maintaining the viability of PDL cells on avulsed teeth was used.

The trypan blue exclusion staining technique was chosen because it is quick, easily performed, and distinctively differentiates non-viable cells from viable cells. However, the health of the viable cells and their ability to proliferate cannot be determined from this technique. The aim of the present study was only to investigate the effects of experimental solutions on viability of PDL cells.

Up to now, several studies have examined the usefulness of several storage media. Results indicated tap water, saliva, and saline are all ineffective in maintaining PDL cell viability, therefore, they are no longer recommended as storage media because of their hypotonic properties and high incidence of bacterial contamination leading to the rapid death of the PDL cells.[26]

HBSS is a standard saline solution that is widely used in biomedical research to support the growth of many cell types.[27] It is non-toxic, pH-balanced, and contains many essential nutrients.[28,29] A tooth preservation system utilizing HBSS as a storage medium is commercially available as Save-A-Tooth™ (Save-A-Tooth Inc., Pottstown, PA, USA),[27] although it is not yet widely available in pharmacies.

S. officinalis extracts have never been tested for preserving PDL cell viability up to now. In this study four different concentrations of *S. officinalis* extracts in PBS were compared to HBSS and tap water. Two point five percent *S. officinalis* showed better results than HBSS, PBS, and tap water at all times except at 1 hour, where there was no significant difference between *S. officinalis* and HBSS or PBS.

Oxygen radicals and oxygen tension have been reported to modulate osteoblastic and osteoclastic activities.[30,31] Buttke et al.[32] stated low levels of H_2O_2 in media used for storing avulsed teeth might adversely affect cells of the attachment apparatus. Oxidative damage may promote root surface resorption via toxic effects on mechanically damaged cells of the PDL or cementum or by enhancing the resorptive activity of clastic cells. They also suggested storing avulsed teeth in a medium containing one or more antioxidants might increase replantation success. It is well known the antioxidant activities of *Salvia* species are due to the beneficial properties of their phenolic constituents. They have been shown to have antioxidant activity, and it is likely the activity of the examined plants is due to these compounds.[33] Flavonoids are powerful antioxidants, and they have been shown to be capable of scavenging free radicals and thereby protecting against lipid peroxidation in the cell membrane. Antioxidant activity of *S. officinalis* is due to its phenolic contents as rosmarinic acid, carnosic acid, carnosic acid, salvianolic acid, and its derivatives carnosol, rosmanol, epirosmanol, rosmadial, and methyl carnosate.[34-36]



Several studies showed favorable effects of either topical or systemic antibiotics on the prevention of root resorption after replantation.[37-39] The use of systemic penicillin after an avulsion injury has been recommended to decrease the occurrence of resorption complications. Tetracycline has been reported to possess anti-resorptive properties in addition to their anti-microbial actions.[37] Previous studies have indicated inflammatory resorption and ankylosis, which are frequent sequela after delayed tooth replantation, can be greatly reduced by treating the root surface with 1% solutions of stannous fluoride and tetracycline.[40] Antimicrobial activities

of polar extracts of *Salvia* species could be attributed to the presence of several types of compounds belonging to different classes such as oleoresins,[41] sterols and their derivatives, flavones and flavonoids,[42] and more polar thermo labile and/or thermo stable phenolics in the hydrophilic subfractions of methanol extract.[43] Since *S. officinalis* has antimicrobial properties, this can make propolis a favorable storage media.

Based on the favorable results obtained in the present study a medium containing *S. officinalis* can be recommended as a suitable transport medium for avulsed teeth. *S. officinalis* not only keeps PDL cells alive but also has antimicrobial, anti-inflammatory, and antioxidant properties. Because storage media containing *S. officinalis* are not available, HBSS is still the first choice.

Conclusion

S. officinalis can be recommended as a suitable transport medium for avulsed teeth.

Clinical Significance

The findings of this study support the use of *S. officinalis* as another option for clinicians to use to store and transport avulsed teeth until reimplantation procedures can be done.

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