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ORIGINAL ARTICLE

Dynamic assessment of *Capparis spinosa* buds on survival of periodontal ligament cells using a real-time cell analysis method

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Abstract

Background: Tooth avulsion is the most severe type of traumatic dental injuries and it results in the complete displacement of the tooth out of its socket in alveolar bone. Reimplantation of the tooth is considered to be a best treatment modality due to its biological and psychological advantages. Its prognosis depends on the extra alveolar time, the storage medium, and the patient's general health.

Objective: The aim of this study was to evaluate the effect of *Capparis spinosa* (*C. spinosa*) in maintaining the viability of human periodontal ligament (PDL) cells using a real-time cell analysis method.

Materials and Methods: Periodontal ligament cells were obtained from healthy human third molars extracted for orthodontic purposes. The storage media tested were: Dulbecco's Modified Eagle Medium (DMEM), *C. spinosa,* Hank's Balanced Salt Solution (HBSS), and light milk. A real-time cell analyzer system was used to evaluate cell viability. After seeding cell suspensions into the wells of the E-plate 96, PDL cells were treated with each of tested media and monitored for every 5 min for 26 h. Statistical analysis of the data was accomplished using one-way analysis of variance complemented by the Tukey test. The level of significance was set at $P < 0.05$.

Results: Dulbecco's Modified Eagle Medium (control) and *C. spinosa* groups had significantly higher cell index values compared with the HBSS and light milk ($P < 0.05$). Although, *C. spinosa* showed better results than DMEM (control), but this difference was not found statistically significant.

Conclusion: *Capparis spinosa* can be a suitable, alternative storage medium for avulsed teeth.

Key words: Avulsion, *Capparis spinosa*, cell viability, storage media, real time cell analyzer

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Introduction

Avulsion is a complete displacement of a tooth from its alveolar socket as a result of trauma. Treatment protocols should include management of the pulp and the periodontal ligament (PDL) cells, with the latter being far more important, in order to improve the long-term survival and prognosis of the teeth. Because, PDL cells are essential for the healing of replanted avulsed teeth.[1] When avulsion occurs, the tooth should be immediately replanted at the site of the accident to prevent further damage to the PDL cells from desiccation. However, immediate repositioning of teeth is not always possible under certain conditions. In such a case, storage media is used to preserve PDL cell viability. An ideal media must be appropriate pH and physiological osmolarity and should include the presence of nutritional

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substances that allow cell survival.[1] For this reason, the choice of a suitable storage media until the replantation of the avulsed tooth is very important.

Various types of media have been examined as temporary storage media, tap water, saliva, milk, saline, Hank's balanced salt solution (HBSS), Save-A-Tooth System and ViaSpan. Some of other storage media including egg white, powdered milk, Gatorade, and propolis are being recently studied and tested.[2-5]

Capparis spinosa (*C. spinosa*) is one of the most commonly found aromatics in the Mediterranean kitchen. It is native to the Mediterranean region and is also widely grown in the dry regions in west and central Asia. Its immature flower buds, unripe fruits, and shoots are consumed as foods or condiments in cooking.[6] It is used in medicine for its diuretic, constipant and tonic properties.[7] Different parts of this plant, including the flower buds, fruits, seeds, shoots, and bark of roots, were traditionally used as folk medicines in the treatment of disorders, such as rheumatism, stomach problems, headache, and toothache.[8] Previous chemical studies on *C. spinosa* have reported the presence of alkaloids, lipids, flavonoids, polyprenols, and aliphatic glucosinolates,[9-11] which are naturally occurring products belonging to the order Capparales, known as flavor compounds, cancer preventing agents, antioxidants, and biopesticides.[12] The aim of this study was to evaluate the effect of *C. spinosa* in maintaining the viability of human PDL cells by using a real-time cell analysis method.

Materials and Methods

Extraction of *Capparis spinosa* buds

The capers (flower buds of *C. spinosa*, obtained from Gaziantep, Turkey, and collected in June 2012) were washed with hot water 3 times, chopped into small pieces. They were extracted with methanol (100 gr of pieces mixed with 500 mL 96% methyl alcohol for each socket) at 60–80°C for 12 h by using a Soxhlet device (Gerhardt EV 14C; Gerhardt Inc., Brackley, Northants, UK). Extracts filtered by Whatman no: 4 filter, and then evaporated under high vacuum at 40°C by a rotary evaporator (Heildolph Digital/HB 57; Albany, NY, USA) to remove methyl alcohol. Extracts stored at +4°C till experiment.

Primary culture of periodontal ligament cells

The outline of this study was approved by the Ethics in Clinical Research Committee of Gulhane Military Hospital, Turkey. PDL cells were obtained from healthy human third molars extracted for orthodontic purposes. The teeth were extracted as atraumatically as possible and washed in sterile saline solution to eliminate the residual blood. PDL tissues were scraped with a size 15 scalpel using aseptic techniques and then transferred to the culture medium.

Periodontal ligament samples were washed twice with HBSS, and placed into tissue culture flasks (25 cm^2). The explants were incubated with culture medium consisting of Dulbecco's modified Eagle's Medium (DMEM), 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, glucose (4.5 g/L), NaHCO3 (3.7 g/L), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin (2.5 mg/mL; all from Biochrom KG, Berlin, Germany) supplemented with 10% heat inactivated fetal calf serum (Pan Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidified atmosphere of 10% CO_2 in air.

Culture medium was renewed twice per week until cells reached confluency. For subcultivation, cells were detached from the culture flasks with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Sigma, Saint Luis, Missouri, USA) for 3–5 min. Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages. Cell morphology was visualized with phase contrast microscopy (TNM, Nikon, Tokyo, Japan).

Experimental groups

Cells were washed by phosphate buffered saline (PBS), and these cells were exposed to different experimental media. The storage media used were as follows: Group 1. DMEM as a control, Group 2. *C. spinosa* (10 mg/ml), Group 3. HBSS, Group 4. Light milk (UHT skimmed milk with 0.12% fat content; Pinar Dairy Products Inc., Istanbul, Turkey).

Real-time cell analysis

The xCELLigence system (Roche Diagnostics, Mannheim, Germany and ACEA Biosciences, San Diego, CA, USA) was used for real-time cell analysis according to the instructions of the supplier. It consists of four main components: The impedance based real-time cell analyzer (RTCA), the RTCA single plate (SP) station, the RTCA computer with integrated software, and disposable E-plate 96. RTCA SP station fits inside a standard tissue culture incubator, while an analyzer and laptop computer with software will be on the outside. The core of the xCELLigence system is the E-plate 96: This is a single use, disposable device used for performing cell-based assays on the RTCA SP instrument, which has similar application like commonly used 96-well micro titer plate. However the E-plate 96 differs from standard 96-well micro titer plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells inside each well to be monitored and assayed. The E-plate 96 has a low evaporation lid design: The bottom diameter of each well is 5.0 ± 0.05 mm; with a total volume of 243 ± 5 μL, approximately 80% of the bottom areas of each well is covered by the circle-on-line electrodes, which is designed to be used in an environment of +15 to +40°C, relative humidity 98% maximum without condensation.[13]

The electronic impedance of sensor electrodes is measured to allow monitoring and detection of physiological

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changes of the cells on the electrodes. The voltage applied to the electrodes during RTCA measurement is about 20 mV (RMS). The impedance measured between electrodes in an individual well depends on electrode geometry, ion concentration in the well and whether or not cells are attached to the electrodes. In the absence of cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance.[13] Thus, the more cells that are growing on the electrodes, the larger the value of electrode impedance.

Cell growth and proliferation assay

Periodontal ligament cells were grown and expanded in tissue-culture flasks. After reaching ~ 75% confluence, the PDL cells (passage 9) were washed with PBS, afterwards detached from the flasks by a brief treatment with trypsin/EDTA. Subsequently, 50 µL of cell culture media at room temperature was added into each well of E-plate 96. After this the E-plate 96 was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured during 24 h. Meanwhile, the cells were resuspended in cell culture medium and adjusted to 200 cells/µmL. 10 µL of each cell suspension was added to the 190 µL storage media containing wells on plate. After 30 min incubation at room temperature, plate was placed into the cell culture incubator. Finally, adhesion, growth and proliferation of the cells was monitored every 5 min for a period of up to 26 h and 30 min via the incorporated sensor electrode arrays of the E-Plate 96.

Statistical analysis

All statistical procedures were performed using a statistical software program (SPSS v17.0; SPSS Inc., Chicago, IL, USA). Statistical analyses were carried out using one-way analysis of variance and *post-hoc* multiple comparisons Tukey HSD tests. Statistical significance was set at $P < 0.05$.

Results

Table 1 represented the means and standard deviations for all the groups. DMEM (control) and C. *spinosa* groups had significantly higher cell index values compared with the HBSS and light milk ($P < 0.05$). However, no significant difference was found between the DMEM (control) and C. *spinosa* groups ($P > 0.05$). As shown in Figure 1, cell index values of C. *spinosa* increased regularly thorough the study. DMEM showed best score at time 8 h afterwards viability value was decreased. Still it showed best results among other test solutions-light milk and HBSS. On the other hand, none of the tested media were found toxic to cells since negative values were not detected. Increased viability values of C. *spinosa* and DMEM could be correlated according to their proliferative effect. Among all test media, C. *spinosa* showed the best result for cell viability for all the time periods.

Table 1: Mean±SD of the cell index values in each group and statistical differences

Groups	Cell index values			
	Mean	SD	Minimum	Maximum
DMEM (control)	2.41[a]	0.89	−0.12	3.44
C. Spinosa	2.53[a]	0.86	0.00	3.41
HBSS	0.15[b]	0.02	0.00	0.20
Light milk	0.33[c]	0.11	0.00	0.50

Groups with same lower case superscripts are not significantly different ($P>0.05$). SD=Standard deviation; DMEM=Dulbecco's modified Eagle medium; *C. spinosa*=*Capparis spinosa*; HBSS=Hank's balanced salt solution

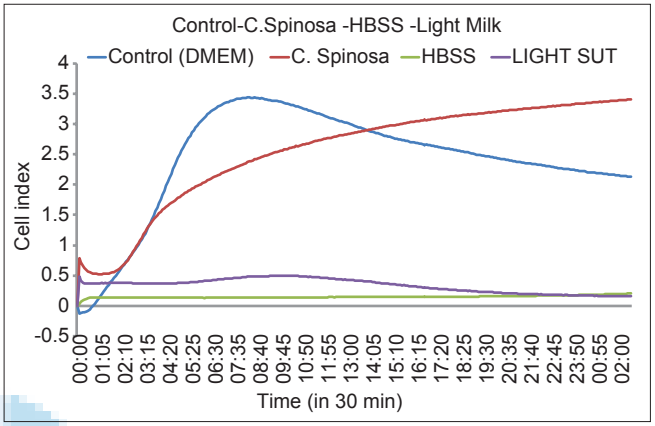


Figure 1: Dynamic monitoring of cell viability for 26 h of tested storage media

Discussion

To minimize the future complications such as ankylosis and root resorption after the avulsion injury, the avulsed teeth should be stored in a temporary medium capable of maintaining PDL cell viability.[14] Considering the critical role of these media, an informed choice of a suitable medium is essential for a successful outcome. In geeral, milk and HBSS have been recommended to be used as storage media for avulsed tooth. Milk has been shown to maintain human PDL fibroblast viability and is accepted by the American Association of Endodontists as a suitable transport media.[1,15,16] Several authors that researched the viability of PDL cells in milk storage medium have reported 70–90% survival rates and low frequency of root resorption after different time periods.[4,5,17-20] There is evidence that milk with a lower fat content may be more appropriate at maintaining cell viability than milk with a higher fat content.[5,15] Milk can usually be obtained on short notice, but even 10 min of desiccation can affect the outcome of replantation.[21] It prevents cell death but does not restore the cells' normal morphology and ability

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to differentiate and undergo mitosis.[1,22] Another storage medium is named HBSS. It is a standard saline solution that is widely used in biomedical research to support the growth of many cell types.[23] It is nontoxic, pH balanced, and contains many essential nutrients. A tooth-preserving system using HBSS as a storage medium was developed and has become commercially available. HBSS does not need to be refrigerated, and it can preserve an avulsed tooth.[3] According to the a recent study[19] the storage time of HBSS had a negative influence on its ability to maintain PDL viability. de Souza *et al.*[19] showed that the milk (light or whole) had the greatest capacity to maintain PDL viability when compared with coconut water, HBSS, and tap water. In the present study, long-shelf life milk with a lower content has been used. It was chosen, because it can be readily available in locations where avulsions usually occur. Results of the study showed that the cell index values of the light milk and HBSS were significantly lower compared with the DMEM and *C. spinosa* groups. They protected the cells viability but did not show any proliferative effect with time [Figure 1].

Dulbecco's Modified Eagle Medium, which contains approximately 4 times as much of the vitamins and amino acids present in the regular Eagle's modified essential medium formulation and 2–4 times as much glucose. In addition, it contains iron and phenol red. DMEM is suitable for most types of cells.[24] However, it is not available to the public and therefore of little value as a storage medium for avulsed teeth.[21] We used DMEM as a control group to compare with the other test media.

Capparis spinosa is one of the most commonly found aromatics in the Mediterranean kitchen, and it is also important in the commercial preparation of frozen food. The aromatic part of the caper is the floral bud, which is gathered just before it blossoms. The plant is typically not cultivated, but rather the wild buds are harvested by seasonal pickers. Before commercial packaging, the buds are stored under salt. Chemical studies on *C. spinosa* have reported the presence of alkaloids, lipids, flavonoids, and glucosinolates, which are naturally occurring products belonging to the order Capparales, known as flavor compounds, cancer preventing agents, and biopesticides.[11,12] Previous studies[25-27] stated that the methanolic extract from *C. spinosa* buds exhibited good antioxidant activities. Their results suggest that the antioxidant activities of the methanolic extract are related to the high level of phenolic compounds, and other flavonoid compounds such as kaempferol and quercetin.[10,11,28] Some studies[29,30] also stated that *C. spinosa* has strong antiinflammatory effect.

Oxygen radicals and oxygen tension have been reported to the modulate osteoblast and osteoclast activities.[31] Buttke and Trope[32] stated that low levels of hydrogen peroxide 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.[32] Storing avulsed teeth in a medium containing one or more antioxidants might increase replantation success. Until now, studies revealed that *C. spinosa* has a strong antioxidant capacity.[10,11,25-27,33] One of the major component of *C. spinosa* is flavonoids. They are powerful antioxidants, and they have been shown to be capable of scavenging-free radicals and thereby protecting against lipid per oxidation in the cell membrane.

Until date, there has been no study which evaluated the effect of *C. spinosa* on PDL cell viability in avulsed tooth cases. It has exhibited higher cell index values compared with the DMEM, HBSS, and light milk. Cell index values of *C. spinosa* were increased with time. Interestingly, cell index values of DMEM were decreased with time after 8th h. Increased viability values of *C. spinosa* and DMEM different from the HBSS and light milk could be correlated according to their proliferative effect.

The xCELLigence technology measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate) or at the bottom side of a micro porous membrane (CIM16-plate). These changes are caused by the gradual increase of electrode surface occupation by (proliferated/migrated/invaded) cells during the course of time. This method of quantitation is directly proportional to cellular morphology, spreading, ruffling and adhesion quality as well as cell number.[34,35] Different biocompatibility test methods such as cell growth, mitochondrial dehydrogenase of active cells and changes in metabolic activity have been used to evaluate the viability of PDL cells for different transport media.[21] We conducted experiments with a new real-time cell analyze system. Compared with conventional endpoint cell-based assays, dynamic monitoring of cell response, such as cell adhesion, spreading, proliferation, and cell death, is an advantage of the real-time system to optimize the cell concentration for *in vitro* assays. It also allows both cell and assay conditions to be constantly obtained before and during the experiments, and is generally less labor-intensive, provides kinetic information on the studied processes and does not affect cell viability, potentially generating further experimentation possibilities.[36,37] Furthermore, the response of living cells to, for example, a chemical exposure can be monitored in real time; this is impossible with current end-point assays.[33]

Conclusion

Based on the results obtained in this study, *C. spinosa* might be recommended as a suitable storage media for avulsed teeth. It not only keeps PDL cells alive but also has anti-inflammatory and anti-oxidant abilities. These benefits

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of *C. spinosa* make it very favorable. New studies, using animals are needed to research the effects of *C. spinosa* on replanted teeth clearly.

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