# Evaluation of in vitro effect of *Morus rubra* (red mulberry) on survival of periodontal ligament cells

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***Objective.*** The purpose of this study was to determine the ability of the juice of *Morus rubra* fruit to serve as a temporary storage medium for the maintenance of periodontal ligament (PDL) cell viability of avulsed teeth.
***Study design.*** PDL cells were obtained from healthy third molars and cultured in Dulbecco's Modified Eagle's Medium (DMEM). Cultures were subjected to 4.0%, 2.5%, 1.5%, and 0.5% of the juice of *M. rubra* fruit, Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), and tap water. Tissue culture plates were incubated with experimental media at 37°C for 1, 3, 6, 12, or 24 hours. PDL cell viability was assessed by trypan blue exclusion. Statistical analysis of the data was accomplished using 1-way analysis of variance complemented by the Tukey test. The level of significance was 5% ($P < .05$).
***Results.*** The efficacy of 4.0% and 2.5% *M. rubra* at 3, 6, and 12 hours was found to be significantly better than HBSS ($P < .05$). At 24 hours, 4% *M. rubra* was found to be similar to HBSS, but 2.5% *M. rubra* was found to be significantly worse than HBSS ($P < .005$). The results showed that juice of the fruit sample of *M. rubra* studied at a concentration of 4% was a more effective storage medium than other groups.
***Conclusion.*** Juice of the fruit of *M. rubra* can be recommended as a suitable transport medium for avulsed teeth. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:e66-e69)**

The reported incidence of complete avulsion 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. If the tooth cannot be replanted for any reason, the extraoral dry time must be minimized by placing the tooth in an appropriate storage medium. The optimum storage medium should be able to preserve the viability, mitogenicity, and clonogenic capability of the injured PDL and their progenitors. This is essential in order to quickly repopulate the denuded root surface by PDL and prevent osteoclasts from attaching to this area. Several media have been used for storage of avulsed teeth, such as tap water, saliva, milk, Hank's balanced salt solution (HBSS), ViaSpan, culture medium, and propolis.[1-3]

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The mulberry belongs to the genus *Morus* of the family Moraceae. There are 24 species of *Morus* and 1 subspecies, with at least 100 known varieties. Mulberry is found from temperate to subtropical regions of the northern hemisphere to the tropics of the southern hemisphere; they can grow in a wide range of climatic, topographical and soil conditions. Mulberry is widely spread throughout all regions from the tropics to the subarctic and from sea level to altitudes as high as 4000 m.[4,5]

In Turkey, traditional products such as "mulberry pekmez," "mulberry pestil," and "mulberry kome" are made with the fruits. The red-colored fruits are eaten fresh and are also used in marmalades, juices, liquors, natural dyes, and in the cosmetics industry.[6,7]

The plant has also been used medicinally in Turkey. Local people traditionally believe that deep-colored fruits, especially black and red mulberry fruits, are healthier for the human body.[6] Mulberry fruits can be used as a worming agent, as a remedy for dysentery, and as a laxative, odontalgic, expectorant, hypoglycemic, and emetic.[8]

In view of this information we aimed to compare HBSS with natural product *Morus rubra* on enhancement of PDL cell viability.

## MATERIALS AND METHODS

### Preparation of M. rubra extract

Fresh fruits of *M. rubra* were collected from Osmaniye, Duzici, Sogutlugol Plateau (1000 m), Turkey, at its fruit season (15 August 2006). A 100-g amount of

**Table I.** Mean percentage of cell viability

| *Medium* | *1 h* | *3 h* | *6 h* | *12 h* | *24 h* |
|---|---|---|---|---|---|
| 4.0% *Morus rubra* | 98.00 ± 0.8 | 95.00 ± 1.8 | 70.00 ± 1.9 | 50.00 ± 2.5 | 25.00 ± 1.8 |
| 2.5% *M. rubra* | 98.00 ± 1.1 | 90.00 ± 1.9 | 65.00 ± 1.8 | 45.00 ± 1.8 | 20.00 ± 1.8 |
| 1.5% *M. rubra* | 99.50 ± 0.5 | 85.00 ± 1.8 | 50.00 ± 2.9 | 40.00 ± 2.9 | 15.00 ± 1.8 |
| 0.5% *M. rubra* | 99.50 ± 0.5 | 80.00 ± 1.8 | 45.00 ± 1.8 | 30.00 ± 1.8 | 13.00 ± 1.8 |
| 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 |

*HBSS,* Hank's balanced salt solution; *PBS,* phosphate-buffered saline.

fresh *M. rubra* fruits were used for obtaining the juice. Juice yield of the fruits were determined as 15.08% (vol/wt). After obtaining the juice, it was kept in the dark at +4°C until tested.

### Primary culture of human PDL cells

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 the residual blood. PDL tissues were scraped with a #15 scalpel using aseptic techniques and then transferred to culture medium.

The PDL samples were cut into 1- to 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 Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO), 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic aid (HEPES), glucose (4.5 g/L), $NaHCO_3$ (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 (FCS) (Pan Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidified atmosphere of 10% carbon dioxide 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/EDTA Solution (Sigma) for 3 to 5 minutes. PDL cells between the 7th and 12th passages were used in the experiment to achieve their maximum proliferative potential and homogeneity. All tissue manipulations were done under a sterile class I laminar airflow cabinet. Cell morphology was visualized with 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 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 4.0% *M. rubra*, (2) PBS containing 2.5% *M. rubra*, (3) PBS containing 1.5% *M. rubra*, (4) PBS containing 0.5% *M. rubra*, (5) HBSS, (6) PBS, and (7) tap water.

### Assessing the viability of cells by trypan blue exclusion

After exposure, the medium was removed from the wells after 1, 3, 6, 12, and 24 hours and washed with 2 mL sterile PBS. Then, 100 μL of 0.25% trypsin was added and the plates were incubated at 37°C for 5 to 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. After this time, a 20-μL aliquot was removed and placed under a cover slip on a hemocytometer, and both the viable and the nonviable 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: $UC/TC \times 100$, 1where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells). Each experiment was repeated 4 times.

### Statistical analysis

Statistical analysis of the data was accomplished using 1-way analysis of variance complemented by the Tukey test. The level of significance was 5% ($P < .05$).

## RESULTS

The mean percentage values of cell viability are shown in Table I. The efficacy of 4.0% and 2.5% *M. rubra* at 3, 6, and 12 hours was found significantly better than HBSS ($P < .05$). At 24 hours, 4.0% *M. rubra* was found to be similar to HBSS, but 2.5% *M. rubra* was found to be significantly worse than HBSS ($P < .05$). When *M. rubra* solutions were compared,

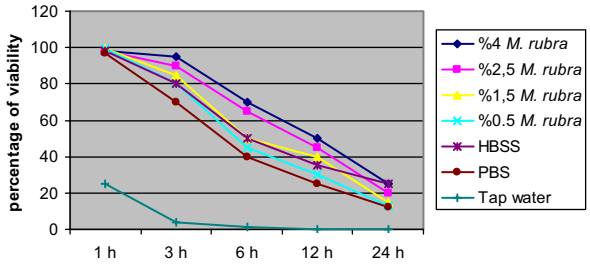



Fig. 1. PDL cell viability.

there was no difference at 1 hour. Whereas, at 3, 6, 12, and 24 hours, 4.0% *M. rubra* was found significantly better than other *M. rubra* solutions. Fig. 1 shows time-dependent results according to experimental solutions on cell viability.

## DISCUSSION

Avulsion injuries are the worst of the dentoalveolar injuries. Usually avulsion involves a single tooth, and the most frequently avulsed tooth is the maxillary central incisor.[9] The 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).[10,11] Clinical studies have shown that inflammatory resorption has an early onset, progresses rapidly, and is associated with pulpal infection, contamination of the root surface, and an extraoral dry time of 15 minutes or more.[10,12-15] Inhibition of inflammatory resorption involves removing the necrotic tissue and filling the root canal with calcium hydroxide.[9] 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.[14-17]

Human PDL fibroblasts behave differently in cell culture than gingival fibroblasts with respect to growth rates and to protein and collagen production.[18] Although lip, gingival, and PDL fibroblasts have similar morphology, they do not behave the same in culture.[3] Protein and collagen production are significantly greater in PDL cells and growth rates differ.[3] Fibroblast cells obtained from the human PDL 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 view of this information, we used a human PDL fibroblast cell culture model for evaluation the efficacy of *M. rubra* extract solutions in maintaining the viability of PDL cells on avulsed teeth.

We chose the trypan blue exclusion staining technique because it is quick, easily performed, and distinctively differentiates nonviable cells from viable cells. However, the health of the viable cells and their ability to proliferate cannot be determined from this technique. We simply aimed 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. Cvek et al.[19] found that avulsed teeth soaked in an isotonic saline for 30 minutes before replantation showed less resorption than those stored dry between 15 and 4 minutes. Lindskog et al.[20] in an in vivo study with monkeys compared saliva with milk and concluded that saliva was less suitable than milk because of its low osmolality and higher risk for bacterial contamination. Matsson et al.[21] examined extracted dog's teeth stored dry for 15, 30, or 60 minutes and then replanted, versus teeth stored dry for the same dry times but also soaked in HBSS for 30 minutes before replantation. These researchers found significantly less resorption on the HBSS-soaked teeth for all dry times. Hilt and Trope[22] found that human lip fibroblasts retained the most viability at 168 hours in ViaSpan versus HBSS, which kept 76% of the fibroblasts vital for 96 hours. They also showed at 6 hours that milk was comparable to HBSS or ViaSpan.

The solution form of *M. rubra* has never been tested for PDL cell viability up to now. In this study, 4 different concentrations of *M. rubra* were compared to HBSS and tap water. The 4.0% and 2.5% *M. rubra* solutions showed better results than HBSS at all hours, except at 24 hours. The 4.0% *M. rubra* solution showed significantly better results than other *M. rubra* solutions and its solvent PBS at all times.

Oxygen radicals and oxygen tension have been reported to modulate osteoblast and osteoclast activities.[23,24] Buttke and Trope[25] stated that 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.[25] They also suggested that storing avulsed teeth in a medium containing one or more antioxidants might increase replantation success. As far as our literature survey could ascertain, we could reach no study directly evaluating the antioxidant activity of *M. rubra*. On the other hand, antioxidative activities of *M. nigra* were recorded by several authors.[26-28]

Several studies showed favorable effects of antibiotics either topical or systemic on avoiding root resorption after replantation. The use of systemic penicillin after an avulsion injury has been recommended to decrease the occurrence of resorption complications. Tetracycline antibiotics have been reported to possess antiresorptive properties in addition to their antimicrobial actions.[29] Previous studies have indicated that inflamma-

tory resorption and ankylosis, which are frequent sequelae after delayed tooth replantation, can be greatly reduced by treating the root surface with 1% solutions of stannous fluoride and tetracycline.[30] There was no record for the antimicrobial properties of *M. rubra* in the literature. This is a deficiency of *M. rubra* that has no antimicrobial efficacy.

Based on the favorable results obtained in this study, *M. rubra* can be recommended as a suitable transport medium for avulsed teeth. However, storage media containing *M. rubra* are not yet available, so HBSS is still the first choice. As far as our literature survey could ascertain, we could find no report concerning the biological properties of the plant species evaluated here; this study could be assumed as the first report on this topic. Results obtained here give us hope to bring new, more efficient and alternative ways on the survival of PDL cells. On the other hand, our results need to be improved by in vivo and in vitro complementary test systems to reveal any other excellent biological properties.

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