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# Evaluation of the long-shelf life honey milk As a storage media for preservation of avulsed teeth

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## Abstract

**Introduction:** Tooth avulsion is defined as the complete displacement of the tooth from its alveolar socket which causes damage to the periodontal ligament structure, cementum, alveolar bone, gingiva, and dental pulp. The purpose of this study was to determine the ability of long-shelf life honey milk to serve as a temporary storage medium for the maintenance of periodontal ligament (PDL) cell viability on avulsed teeth.

**Methods:** PDL cells were obtained from premolars extracted for orthodontic purposes which were clinically healthy and had healthy gingiva (*i.e.* not inflamed).Then, $8\times10^3$ cells were seeded in each well of 96-well plate. and Afterwards treated with long-shelf life milk and honey milk, Hank's Balanced Salt Solution (HBSS) and fresh milk. Different incubation periods were 1, 3, 6, and 9 hours. Dulbecco's Modified Eagle Medium (DMEM) and dry medium were considered as positive and negative control media, respectively. Cell viability was determined by using the MTT (Thiazolyl Blue Tetrazolium Bromide) assay. Data were statistically analyzed with one-way anova, two-way anova and post hoc Scheffe tests. A level of $p\leq0.05$ was accepted as statistically significant

**Results:** The results indicate that all media performed significantly better in maintaining PDL cell viability than the negative control at all time periods. ($p\leq0.001$) After 9 hours, Percentage of viable PDL cells in long-shelf life honey milk, long-shelf life milk and HBSS were 82±0.82, 75±8.13 and 87±2.78 respectively. Furthermore cells' viability in both long-shelf life honey milk and HBSS was significantly better than fresh milk medium ($p=0.003$). Moreover, the results of One-way ANOVA showed long-shelf life honey milk were more effective in preserving the PDL cell viability as well as HBSS after 9 hours.

**Conclusions:** According to the study results, long-shelf life honey milk considered as appropriate storage media which are comparable to HBSS. These media are not only able to keep more cells viability after 9h compared to the expensive commercial solutions, but are also be easily accessible.

**Keywords:** Tooth avulsion, Fibroblasts, DMEM, Storage media

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Honey milk A new storage media for avulsed teeth

# بررسی توانایی شیرعسل با ماندگاری بالا در زنده نگه داشتن سلولهای لیگامان پریودنتال

## چکیده

**مقدمه:** بیرون افتادن دندان به معنای جابجایی کامل دندان از حفره دندانی است که باعث آسیب به ساختارهای لیگامان پریودونتال ،سمان ، استخوان آلوئول ،لثه و پالپ دندان می شود. هدف از انجام این مطالعه بررسی توانایی شیرعسل بلند مدت در زنده نگه داشتن سلولهای لیگامان پریودنتال می باشد.

**مواد و روش ها:** برای تهیه سلولهای لیگامان پریودونتال از دندان های پرمولر سالم که به دلیل درمان ارتودنسی کشیده شده بودند استفاده شد.بافت پریودونتالی در این افراد سالم و فاقد هرگونه التهاب بود.سلولها ی لیگامان پریودونتال به تعداد $۸\times۱۰^{۳}$ سلول درمحیط کشت DMEM(Dulbecco's Modified Eagle Medium ) در پلیت ۹۶-well کشت داده شدند تا زمان اتصال سلولها به پلیت، در ۳۷ درجه انکوبه شدند. بعد از آن محیط کشت DMEM را برداشته و به هر well، به میزان ۱۰۰ میکرو لیتر از محیط های شیر مدت دار ، شیر معمولی ، Hank's Balanced Salt Solution (HBSS) و شیر عسل مدت دار اضافه شد. DMEM به عنوان محیط کنترل مثبت و محیط خشک به عنوان محیط کنترل منفی در نظر گرفته شد. سپس پلیت ها به مدت ۱، ۳، ۶، ۹ ساعت در انکوباتور $۳۷^{\circ}$ قرار داده شدند. زنده ماندن سلولها با استفاده از روش MTT(Thiazolyl Blue Tetrazolium Bromide) ارزیابی شد. یافته های مطالعه با استفاده از روش های آماریTwo Way، One Way Anova، Anova، Post hoc scheffe مورد بررسی قرار گرفت.

**یافته ها:** نتایج مطالعه نشان می دهد که میزان بقای سلولهای PDL در تمام محیط ها در تمام زمان هابه صورت معنی داری نسبت به محیط کنترل منفی بهتر می باشد($p\leq0.001$) بعد از گذشت ۹ ساعت میانگین بقای سلولهای لیگامان پریودنتال در محیطهای شیرعسل مدت دار،شیر مدت دار و HBSS به ترتیب ۸۲±۰/۸۲ ،۷۵±۸/۱۳، ۸۷±۲/۷۸ بود. بعد از گذشت ۹ ساعت در هردو محیط شیر عسل و HBSS نسبت به محیط شیرمعمولی به صورت معنی داری میزان بقای سلولها بیشتر بود. ($p=0.003$) نتایج آنالیز One Way Anova نشان می دهد که پس از گذشت ۹ ساعت میزان بقای سلولهای PDL در محیط شیر عسل قابل مقایسه با محیط HBSS بود.

**نتیجه گیری:** با توجه به نتایج مطالعه ی حاضر شیر عسل با ماندگاری بالا و همچنین شیر مدت دار به عنوان یک محیط حد واسط مطلوب که قابل مقایسه با HBSS می باشند در نظر گرفته می شود. این محیط ها علاوه بر اینکه در مدت زمان ذکر شده دارای توانایی برابر در زنده نگه داشتن سلولها در مقایسه با محلولهای تجاری گران قیمت می باشند به راحتی در دسترس بوده و قابل استفاده می باشند.

**واژگان کلیدی:** فیبروبلاست، DMEM، لیگامان پریودنتال

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## Introduction

Tooth avulsion is a complex traumatic injury characterized by severe damage to the periodontal and pulpal tissues. It most frequently occurs in boys, moreover, 8-12 years old children are more affected. This is probably due to low mineralized alveolar bone and loose periodontal ligament (1, 2).

The prevalence of avulsion is 1-16% of traumatic injuries in the permanent dentition **(**3, 4**).** Because the viability of the PDL cells will determine the prognosis for the retention of the replanted tooth, a suitable transport medium is extremely important. It has been shown that storing the tooth in a proper media is more important than the extra alveolar period (5-7).

Up to now, several known storage media, such as saliva (buccal vestibule), saline, milk, culture media, Hank's Balanced Salt Solution (HBSS) and Viaspan, have been examined and some media have been tested recently (*i.e.* coconut water, propolis, egg albumen, Gatorade, green tea **(**4, 5, 7). Although HBSS, ViaSpan and Eagle's medium have great potential to maintain the PDL cells in a viable state after avulsion, the practicalities of using these solutions, the costs and the lack of ready availability to the general public make them less than ideal.

Milk remains the most convenient, cheapest and readily available solution in most situations while also being capable of keeping PDL cells alive. Hence, milk remains the storage medium of choice for avulsed teeth that cannot be replanted immediately or very soon after the avulsion. Appropriate osmolality and pH for optimal growth of cells accompanied by the presence of nutritional substances in milk may be responsible for its acceptable results as storage media (6-10).

Regular pasteurized milk has a short shelf-life and requires refrigeration, which makes it less likely to be immediately available in a trauma prone setting. The potential of honey to assist with wound healing has been demonstrated repeatedly (9).

It has proven valuable in the treatment of infantile gastroenteritis, infected surgical wounds, burns, decubitus ulcers, skin crafting and. It has been shown that natural unheated honey has some broad-spectrum antibacterial activity when tested against pathogenic bacteria, oral bacteria as well as food spoilage bacteria (10). long-shelf life honey milk contains have minimally 8% nonfat solid milk, 3gr protein, 11gr carbohydrate, 0.1gr calcium, 0.6gr minerals and 0.12gr phosphorous and natural honey (5%).

This product have extended storage capability of at least 6 months without the need for refrigeration. To date, there have not been any published studies examining the use of honey milk in iran. If honey milk products were shown to be effective at maintaining PDL cell viability, they could be recommended as suitable transport media, with the advantage of increased availability in trauma prone settings.

The purpose of this study was to determine the ability of Iranian long-shelf life honey milk to serve as a temporary storage medium for the maintenance of periodontal ligament (PDL) cell viability on avulsed teeth.

## Methods

PDL cells were obtained from premolars extracted for orthodontic purposes which were clinically healthy and had healthy gingiva (*10 to 12 year –old children*). The tooth was washed with normal saline for 3 times and transferred to the laboratory in test tubes containing 10 ml DMEM culture medium (Biosera, South Africa) in 30 minutes.

Then, the cells of the middle one-third region of the root surface were separated from the tooth surface using a sterile surgical blade. After washing the separated tissue, it was divided into smaller pieces, turned into cell suspension using collagenase (4 mg/ml) and dispase (3 mg/ml), passed from a strainer, and cultured in Dulbecco's Modified Eagle Medium (DMEM ) culture medium containing 10% FBS (Biosera), 10% AB-Serum, 100 U/ml penicillin, 100 U/ml streptomycin (PAA), and 1% antimycotic at 37°C, 95% air, and 5% $Co_2$. 3 to 4 times passages were performed. In the present study, $8\times10^3$ cells were seeded in each well of the 96-well plate.

Each experimental storage media were repeated for 6 times. Different incubation periods were 1, 3, 6, and 9 hours. The plates were incubated overnight. After that, the culture media was replaced with 100 µL of the 5 different groups for the experimental time intervals (1, 3, 6, 9 hours).

1-honey milk with 2% fat (Mihan Corp Tehran-Iran)

2- fresh milk with 2.5% fat (Apada Corp Shiraz–Iran)

3-DMEM culture medium (positive control)

4-dry medium (negative control) and

5-HBSS culture medium (Biosera-XC-S2065)



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6- long shelf-life milk (Mihan Corp Tehran-Iran). In this study, long-shelf life milk and long-shelf life Honey milk volume was 200 cc.

The viability of the cells was determined by MTT assay; that is, 150 µL of MTT (20µL/mL) was added and cultures were then incubated for 4 hours and were replaced with 150 µL of Dimethyl Sulfoxide (DMSO). Afterwards optical density was measured at 492 nm with ELISA plate reader.

pH determinations were accomplished on the test solutions using an Orion pH Meter model 720A (Orion Research, Inc. Boston, MA). Osmolality measurements were performed with a Vapro model 5520 Vapor Pressure Osmometer calibrated from 100 to 500 mOsm/kg (Wescor, Inc. Logan, UT). The results were statistically analyzed using SPSS 19 software program (SPSS Inc. Chicago, IL, USA). Data were statistically analyzed with two-way anova test to assess the effects of different media (groups) and time intervals on cell viability.

A post hoc Scheffe test was then used to evaluate the probable differences among groups. For comparing the groups more details, one-way anova test is employed in each time interval. A level of $p \leq 0.05$ was accepted as statistically significant.

## Results

The mean of PDL cell viability, for each media and for the positive and negative controls are shown in table1. The results indicate that all media performed significantly ($p \leq 0.001$) better in maintaining PDL cell viability than the negative control at all time periods. Results of one-way anova test show that HBSS and honey milk preserve cell vitality as good as positive control (DMEM) at all experimental time periods. After 9 hours, the cells' viability in honey milk, HBSS and DMEM was significantly better than fresh milk medium ($p=0.003$). Moreover, the results of One-way ANOVA showed honey milk were more effective in preserving the PDL cell viability than the long shelf-life milk after 9 hours. The pH and osmolality measurements reported in table2 reveal that even though the experimental solutions had similar pH and osmolality values. Figure 1 shows the viability PDL cells in different times and different mediums.

**Table 1. Percentage of viable PDL cells [Mean (SE)] in different storage media at several time points**

| Storage media | 1 hour (Mean±SD) | 3 hours (Mean±SD) | 6 hours (Mean±SD) | 9 hours (Mean±SD) |
|---|---|---|---|---|
| DMEM | 100±3.07 | 100±2.23 | 100±2.52 | 100±3.05 |
| apada Milk | 94±2.9 | 75±2.23 | 57±3.05 | 50±6.09 |
| mihan Milk | 96±2.14 | 94.86±1.19 | 83±3.07 | 75±8.13 |
| honey milk | 92±4.5 | 85±2.33 | 78±2.91 | 82±0.82 |
| HBSS | 94±1.97 | 95±0.48 | 87±1.07 | 87±2.78 |
| Negative control | 38±4.02 | 35±3.05 | 30±2.23 | 27±1.28 |

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS) *Negative Control: Dry condition*

**Table 2. pH and osmolality of transport media**

| Medium | pH | osmolality(mosm/L) |
|---|---|---|
| DMEM | 6.87 | 310 |
| Fresh Milk | 6.05 | 290 |
| Honey milk | 6.07 | 280 |
| Long Shelf Life Milk | 6.24 | 285 |
| HBSS | 6.75 | 400 |

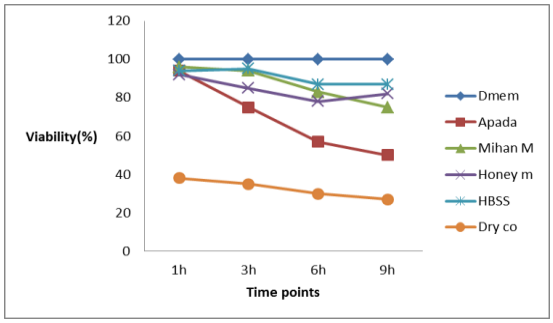



**Figure 1. Graph shows the PDL cell viability**



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## Discussion

The most crucial factor which influences the final prognosis is existence of viable PDL cells. Therefore, immediate replantation is considered as the treatment of choice. If this is not possible, the tooth should be kept in a proper storage media (5, 11).

So ideal treatment is quick replantation of avulsed tooth to prevent more cell necrosis and replacement root resorption (12). Unfortunately, some conditions such as lack of knowledge in public usually postpone the immediate replantation. In such situations, the tooth should be kept in suitable storage media to preserve maximum PDL cells viability before replantation (3).

The results of this study showed that long-shelf life honey milk is an effective storage media for avulsed teeth as HBSS. HBSS is a standard saline solution, which is widely used in biomedical research to support the growth of many cell types. It is non-toxic, pH-balanced, and contains many essential nutrients. HBSS has an osmolality that ranges from 270 to 320 mOsm (13).

Tap water is hypotonic and is not a suitable storage medium. It has been shown that solutions with osmolality in a range of 230–400 mOsm are good for cell growth, and optimal growth will occur in a range of 290–330 mOsm (14). The osmolality of honey milk long-shelf life milk is 280 mOsm.

Physiological pH is another critical factor for PDL cells viability. It has been indicated that cells can survive between pH 6.6 and 7.8 (15). All of the solutions investigated in our study have physiologic pH. The nutritive value and the specific sterilization and homogenization processes of long-shelf life honey milk may be another factor that explain why they preserve PDL cells viable similar to HBSS.The honey milk contains have 8% non fat solid milk, 3gr protein, 11gr carbohydrate, 0.1gr calcium, 0.6gr minerals and 0.12gr phosphorous.

Fresh milks are pasteurized at 75°C for 15 seconds, while long-shelf life ones are pasteurized at 140°C for 3 seconds. Such a high temperature ascertains the ideal inactivation of the bacteria. Honey has been used as a medicine throughout the ages and in more recent times has been "rediscovered" by the medical profession for treatment of burns, infected wounds, and skin ulcers. The large volume of literature reporting its effectiveness indicates that honey has potential for the treatment of periodontal disease, mouth ulcers, and other problems of oral health (16).

Various studies have shown that the capability of milk in keeping the PDL cells viability is reduced after 2-3 hours. Blomlöf et al. introduced milk as an appropriate storage medium for a short period of time (8). In another study, Molan showed that only 50% of the PDL cells remained viable after 12 hours (9).

Moreover, Lekic et al. showed that milk Is effective as well as HBSS; however, its effectiveness reduced after 2 hours (17). In the current study also, fresh milk revealed the best outcome after 1 hour. After 9 hours, long-shelf life honey milk showed better results in comparison to fresh milk. This is in agreement with the study by Marino et al. which showed that after 8 hours, the cells' viability was higher in milk compared to HBSS (18).

Maintenance of viability of PDL cells in the long shelf-life honey milk may be due to the nutrients that are present such as proteins, essential amino acids, vitamins and minerals which help in nourishing the cells and maintaining their viability. In addition based on the favorable results obtained in this study, long shelf life honey milk can be recommended as a suitable transport medium for avulsed teeth. Due to its ease of storage and long shelf life, it can be available in schools, gyms, and outdoor athletic fields, where tooth avulsions are most likely to occur.

## Conclusion

The present study investigated the long-shelf life honey milk and showed this product could protect the avulsed PDL cells up to 9 hours. Not only these media are capable of keeping the cells viable as the expensive commercial solutions, but they can also be easily accessed and used. Yet, another advantage of this product is that they are not required to be kept in the refrigerator which causes them to be easily accessible in the places where dental injuries may more frequently occur.

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