Original Article

Pomegranate Juice (Punica Granatum): A New Storage Medium for Avulsed Teeth

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Abstract

Objective: There is evidence indicating that pomegranate juice contains many of the essential properties necessary to retain cell viability and cell proliferation. These properties indicate that pomegranate juice is a suitable storage medium for avulsed teeth. However, this idea has not yet been tested. In this study, the capacity of pomegranate juice (PJ) as a storage medium for retaining avulsed teeth was evaluated.

Materials and Methods: PDL fibroblasts were obtained from healthy human premolars and cultured in Dulbecco's Modified Eagle's Medium (DMEM). Cultured cells were subjected to different concentrations of pomegranate juice (PJ), 1% Hank`s balanced salt solution (HBSS) and tap water for 1, 3, 6 and 24 hours. PDL cell viability was assessed by the neutral red uptake assay.

Results: The results indicated that 7.5% PJ was the most effective solution for maintaining PDL cell viability amongst all the experimental solutions and time intervals ($P<0.05$). The results also showed that 1% PJ was as effective as HBSS for maintaining PDL cell viability. The amount of cell viability increased with increasing concentration of PJ at all time intervals ($P<0.001$). This effect is suggestive of the proliferative potential of PJ solution.

Conclusion: In conclusion, PJ can be recommended as a suitable transport medium for avulsed teeth.

Key words: Punica Granatum; Tooth Avulsion; Periodontal Ligament; Neutral Red; Cell Culture

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INTRODUCTION

The reported incidence of tooth avulsion ranges from 1 to 16% of all traumatic injuries to the permanent dentition [1]. Studies have shown that a dry period of 2 hours results in necrosis of almost all of the periodontal ligament cells [2]. After avulsion, immediate replantation is the treatment of choice to prevent further damage to the PDL cells from desiccation. However, clinical experience has shown that most avulsed teeth are replanted only after an extended extra-alveolar time, while being

dry or stored in inadequately moist conditions [3]. If the tooth cannot be replanted for any reason, the extraoral dry time must be minimized by placing the tooth in a suitable storage medium [4]. The optimum storage medium should have the ability to preserve the viability, mitogenicity and clonogenic capacity of the injured PDL cells and their progenitors [3]. While HBSS, Viaspan®, milk, normal saline, saliva and tap water [5, 6] are some suggested storage media for an avulsed tooth, recent studies have verified the application of some other storage media with natural sources such as propolis [7], egg white [8], Morus rubra [9], coconut water [10] and green tea extract [11]. Previous studies have indicated that inflammatory resorption and ankylosis are frequent sequelae after tooth replantation [12]. For increasing the replantation success, using a suitable medium with anti-inflammatory, antioxidant and antibacterial properties would be of great advantage [9].

Pomegranate is the fruit of Punica granatum (Punicaceae) that has been extensively used in the folk medicine of many cultures. In traditional medicine, pomegranate fruit has been used to treat acidosis, dysentery, microbial infections, diarrhea, hemorrhage, respiratory pathologies, and hypertension [13]. Besides, pomegranate is a rich source of polyphenolic flavonoids; which possess direct antioxidant properties, such as radical scavenging ability, and indirect antioxidant properties such as induction of endogenous antioxidant enzymes [14, 15]. Pomegranate flavonoids also have anti-inflammatory properties such as restriction of low stimuli activation of inflammation [16]. Moreover, in-vitro studies of pomegranate flavonoids have shown anti-bacterial properties; which relate to gingivitis [17]. Previous studies have indicated that rinsing the mouth with pomegranate extract had the capacity to remove dental plaque bacteria from the teeth [18]. Furthermore, it has been declared that the effects of pomegranate fruit and peel extracts on proliferation and differentiation of osteoblasts significantly improved the pocket depth, level of attached gingiva and bleeding on probing in gingival pockets [19, 20]. To date, PJ extract has never been tested for PDL cell viability. Based on these properties of PJ, which offer the essential features of a good storage medium, this study was designed to evaluate the capability of PJ for this purpose.

MATERIALS AND METHODS

Preparation of Pomegranate Juice

Fresh fruits of pomegranate (scientifically authenticated as Punica granatum) containing red pulps/red peels were collected from Iran (Shiraz) at its fruit season. The amount required for this study was prepared and the grains were separated carefully in order to extract the juice. PJ was prepared by squeezing the grains and filtering the juice. Pure juice was placed in Rotary Flash evaporator until the optimal concentration was obtained.

It was then filtered again by filtering paper to be suitable for passing through the 0.2 µm filter. The prepared PJ was kept in the dark at +4^{o}C until tested.

Primary Culture of Human PDL Cells

PDL cells were obtained from clinically healthy premolar teeth that were extracted for orthodontic purposes from patients under 18 years of age. The teeth were extracted as atraumatically as possible and then immersed in Dulbecco's Modified Eagle's Medium (Gibco, UK) that was supplemented with 10% fetal calf serum and 1% 1x penicillin/streptomycin (Gibco, UK). The samples were washed with saline solution and rinsed twice in phosphate-buffered saline (PBS) to eliminate residual blood.

The PDL tissues were removed from the root area with a #15 scalpel using an aseptic technique. The detached tissues were then immersed in 25 cm^2 culture dishes (Nunc, Roskilde, Denmark); which contained 5ml of culture medium.

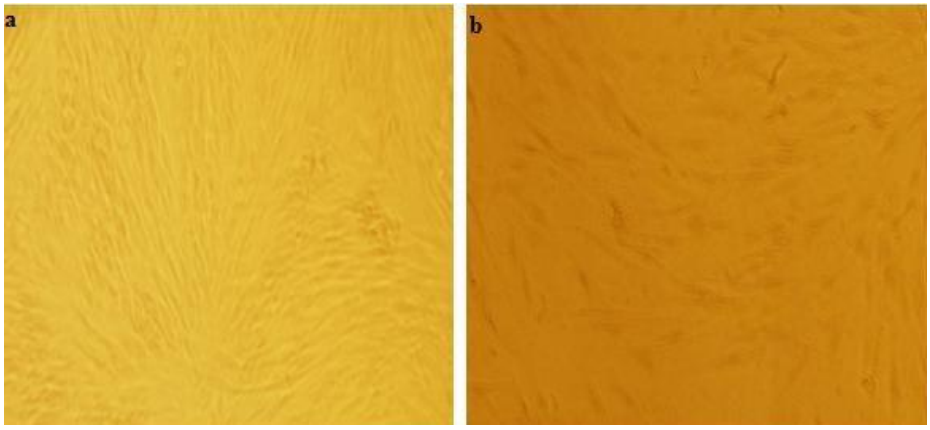

Fig1. Micrograph of PDL cell morphology after 24hrs. a: PJ 1%, b: HBSS 1%

All tissue manipulations were carried out under sterile laminar airflow. The dishes were then incubated at 37°C, 5% CO_2 for an average of 2 weeks in order to observe the HPLF cells (passage zero).

The culture medium was renewed twice per week until the cells reached confluence. The cells were then sub-cultured using 0.25% Trypsin/EDTA (Gibco, UK). Passages number 3-6 were used in this study.

Exposure of PDL Cultures to Different Solutions

HPLF cells from passage 3-6 were cultured in 24-well plates at a density of $4x10^4$ cells /well. After 24 hours, the cells were treated with 1%, 2.5%, 5% and 7.5% PJ solutions; tap water was the negative control and 1% HBSS was the positive control.

A time course study including 1hr, 3hr, 6hr and 24hr time periods was designed and cell viability was assessed at each time point for all tested concentrations using neutral red assay. Cell morphology was visualized with phase contrast microscopy (Leitz, Germany).

Determination of Cell Viability by Neutral Red Assay

The media in each well were aspirated and cells were washed twice with phosphate-buffered saline (PBS); 300 microliters of neutral red was added to each well and plates were incubated at 37° C, 95% O2/5% CO2 conditions for 90 min. The dye was removed and cells were exposed to 300 microliters of solubilizer [Absolute ethanol: 0.1 M Citrate buffer, pH 4.2 (21.01 g citric acid + 200 ml of 1M NaOH per litter [A]); 60 ml of [A] +40 ml 0.1 M HCL mixed 1:1 v/v] after washing. Following 20 minutes of shaking at room temperature, absorbance was measured at 540 nm using ELISA reader. Each experiment was repeated 3 times. The percentage of viable cells was determined by using the following equation: % of cell viability = (mean absorbance of experimental wells/mean absorbance of HBSS wells at 1hour) × 100%.

Statistical Analysis

Differences in cell viability among different tested media during all time intervals were analyzed using -one way repeated measures ANOVA, and multiple comparison was carried out using Tukey's post-hoc test. The level of significance was 5% ($P<0.05$).

RESULTS

Microscopic Examination

Results of microscopic assessment showed that compared to tap water, both HBSS and PJ preserved spindle-like cell morphology even after 24 hours, and PJ was more successful in retaining cell viability and natural cell morphology (Figure 1).

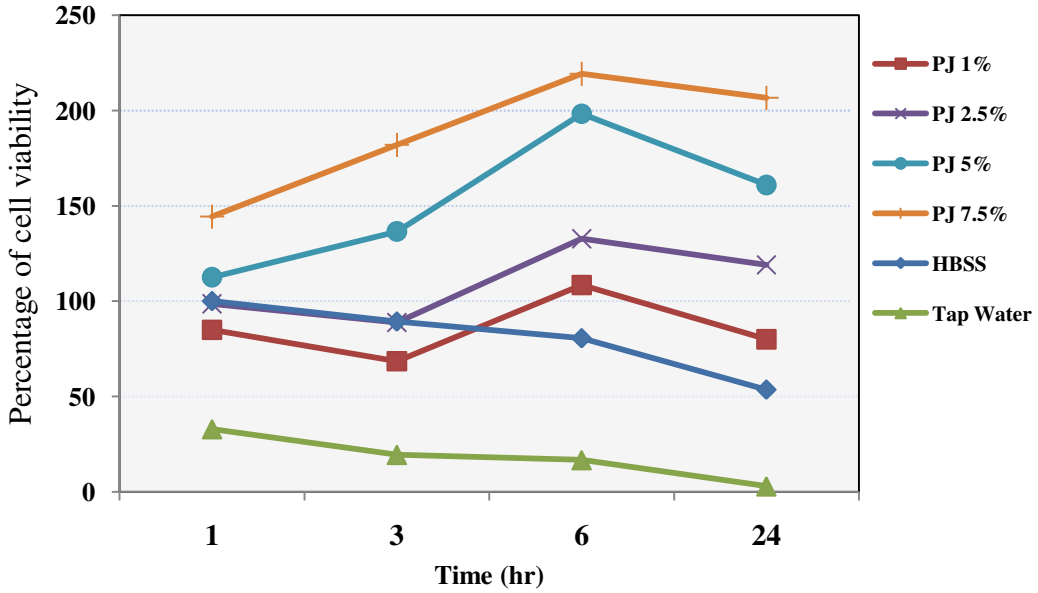

Fig 2. Periodontal ligament cell viability in the tested media at various time intervals

Cell Viability Test by Neutral Red Assay

The mean absorbance, standard deviation and mean percentage values of cell viability in different mediums at various periods are summarized in Table 1. Results revealed statistically significant differences between different tested media and within various time intervals ($P<0.001$).

There was a significant interaction between the tested media and time intervals ($p<0.001$, Figure 2).

The results showed that during all four time intervals, 1% PJ solution performed similarly to HBSS, and the differences were insignificant ($P>0.05$). Across all time intervals, 7.5% PJ solution was found to have the most significant effect on maintaining cell viability compared to HBSS, 1% and 2.5% PJ solutions ($P<0.05$, Table 2).

For comparison of the four PJ experimental groups, repeated measures ANOVA revealed a significant interaction between the time and

Table 1. The mean absorbance, Standard deviation and % of cell viability in tested media at various time intervals

Media	Time (hr)							
	1		3		6		24	
N=3	Mean (SD)	% Viability	Mean (SD)	% Viability	Mean (SD)	% Viability	Mean (SD)	% Viability
PJ 1%	0.29(0.06)	84.95	0.23(0.03)	68.53	0.36(0.05)	108.45	0.27(0.04)	80.03
PJ 2.5%	0.38(0.03)	98.62	0.30(0.04)	88.79	0.45(0.02)	132.74	0.40(0.06)	119.07
PJ 5%	0.49(0.07)	112.58	0.46(0.01)	136.66	0.67(0.01)	198.32	0.54(0.04)	160.96
PJ 7.5%	0.49(0.07)	144.34	0.61(0.01)	182.00	0.74(0.01)	219.27	0.70(0.03)	206.68
HBSS	0.34(0.03)	100.00	0.30(0.05)	89.28	0.27(0.06)	80.53	0.18(0.00)	53.58
Tap water	0.11(0.03)	32.94	0.06(0.00)	19.51	0.05(0.01)	16.73	0.01(0.00)	2.90

PJ: Pomegranate Juice

experimental group factors (P<0.001); although after eliminating the one-hour time point from the time intervals, this interaction was no longer significant (P=0.351).

The results of Tukey's test revealed that at all time intervals, the viability of cells increased with increasing concentration of PJ solution (P<0.001); therefore, 7.5% PJ solution maintained the highest number of vital cells, and this ability decreased with decreasing concentration of PJ solution. At the 6 hr time interval, all four experimental groups had the ability to maintain the highest number of vital PDL cells. Although amongst all concentrations of PJ, the number of vital PDL cells reduced at the 24 hr time interval, this was still significantly higher in 2.5%, 5% and 7.5% concentrations of PJ compared to HBSS (P<0.001).

DISCUSSION

Avulsion is the worst traumatic injury to teeth; which leads to detachment of periodontal ligament from the alveolar socket. The treatment of choice for avulsion is immediate replantation of the tooth. However, due to the lack of knowledge, stress and different conditions at the time of accident, immediate replantation rarely occurs [21, 22].

In this situation, maintenance of PDL vitality until provision of dental treatment is of high importance for a good prognosis.

The prognosis depends on two factors: the interim transport medium and the extra-alveolar time. The capacity of a storage medium to preserve cell vitality is considered more critical than the extra-alveolar time in prevention of inflammation and replacement root resorption [23]. To date, several studies have suggested various substances as storage media [5, 6].

Tap water is not a suitable medium due to its hypotonicity leading to rapid cell lysis. Saliva as a storage medium is more effective than tap water, although it is not widely accepted due to its potential for bacterial contamination [24]; furthermore, saliva is a hypotonic solution which causes cell swelling.

Thus, it may be used as a short-term storage medium, while not being suitable for long-term use [25].

In several studies, milk was identified as a suitable short-term storage medium [8] for up to 48h [26, 21].

HBSS is a widely used standard solution recommended by the International Association of Dental Traumatology as a suitable storage medium for avulsed teeth [1].

Table 2. Comparison between tested media at various time intervals

Groups	PJ1%	PJ2.5%	PJ5%	PJ7.5%	Time
PJ2.5%	P=0.817				1hr
PJ5%	P=0.205	P=0.804			
PJ7.5%	**P<0.001**	**P=0.014**	P=0.116		
HBSS	P=0.754	P=1.000	P=0.860	**P=0.018**	
PJ2.5%	P=0.148				3hrs
PJ5%	**P<0.001**	**P<0.001**			
PJ7.5%	**P<0.001**	**P<0.001**	**P<0.001**		
HBSS	P=0.134	P=1.000	**P<0.001**	**P<0.001**	
PJ2.5%	P=0.117				6hrs
PJ5%	**P<0.001**	**P<0.001**			
PJ7.5%	**P<0.001**	**P<0.001**	P=0.214		
HBSS	P=0.058	**P<0.001**	**P<0.001**	**P<0.001**	
PJ2.5%	**P=0.020**				24hrs
PJ5%	**P<0.001**	**P<0.001**			
PJ7.5%	**P<0.001**	**P<0.001**	**P<0.001**		
HBSS	P=0.159	**P<0.001**	**P<0.001**	**P<0.001**	

Non-significant Significant (P<0.05)

PJ: Pomegranate Juice

Although HBSS has the ability to provide long-term preservation of PDL fibroblasts, a major obstacle in using synthetic storage media is their difficult accessibility during an accident.

This indicates the need to identify other acceptable storage media; which provide a suitable environment for maintenance of PDL cell vitality [27].

Up to now, pomegranate juice has not been tested for preserving PDL cell viability.

In this study, different concentrations of PJ were studied and compared with HBSS and tap water. The results showed a significant difference between HBSS and 7.5% PJ solutions at 1 hr time interval. There was also a significant difference between 2.5%, 5% and 7.5% PJ solutions and HBSS at 6 and 24hr time intervals. This means that the PDL cell viability increases by increasing time and concentration of PJ solution.

According to Figure 2, it can be observed that PJ increased cell proliferation. This indicates the effects of PJ on fibroblast cell proliferation; which resulted in three times more cell viability in 7.5% PJ compared to HBSS after 24 hours. Also, it was observed that cell viability decreased at 1% and 2.5% concentrations of PJ at the 1hr time interval; although a general proliferative effect was observed at 5% and 7.5% concentrations of PJ. This may result from the initial cytotoxicity of PJ; which is followed by proliferative effects at higher doses. Furthermore, cell viability increased in all concentrations of PJ at various time intervals, with the peak effect being observed at the 6hr time point. Buttke and Trope suggested that the storage of avulsed teeth in a medium containing one or more antioxidant components may improve the prognosis of replantation [28]. Nowadays, pomegranate as an ancient fruit is attracting tremendous attention due to its strong antioxidant properties. The potent antioxidant activity of PJ is attributed to its polyphenols including punicalagin, the major fruit ellagitannin and ellagic acid (EA). Punicalagin is the major antioxidant polyphenol ingredient in PJ [29]. Thus, it is assumed to be effective for maintaining PDL cell viability. A previous study suggested that propolis as a storage medium maintained higher viability of PDL cells due to its antibacterial and anti-inflammatory properties [7].

It is known that pomegranate flavonoids have anti-inflammatory and antibacterial properties, while pomegranate polyphenols have antioxidant and antiviral properties which may result in higher viability of PDL cells [13].

An interesting phenomenon observed in our pilot study was that PJ promotes an extremely strong cell attachment for a long duration of time. Although initially trypan blue dye exclusion test was used for determining cell viability, the cells treated with PJ did not detach by trypsin nor collagenase (even with various concentrations and exposure times of the enzymes). Therefore, neutral red assay was preferred. According to a study by Kwak et al, pomegranate contains components such as ellagic acid and punicalagin that are specific inhibitors of beta-secretase (BACE1) and have an inhibitory effect on alpha-secretase (TACE) and other serine proteases such as chymotrypsin**,** trypsin, and elastase [30]. Moreover, in another study that evaluated anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants, it was reported that pomegranate showed 15% anti-elastase and 11% anti-collagenase activity [31]. Strong attachment of cells may occur as a result of serine protease inhibitor components of PJ; which deactivate enzymes such as trypsin and collagenase.

CONCLUSION

In conclusion, the results of this study suggest that pomegranate juice promotes cell viability and induces proliferation of PDL cells.

Therefore, it is recommended to use PJ as a suitable storage medium for avulsed teeth, although further in vitro/ in vivo studies are required in this field.

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