

## EFFECT OF TWO DIFFERENT STORAGE MEDIA OF AVULSED TOOTH ON VIABILITY AND APOPTOSIS OF HUMAN GINGIVAL FIBROBLAST: AN IN VITRO STUDY

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### ABSTRACT

**Objective:** This study aimed to compare the effect of HBSS & almond on viability and apoptosis of human gingival fibroblast in vitro.

**Materials and Methods:** The storage media were initially prepared and diluted in cell culture Dulbecco's modified eagle medium (DMEM) and IC50 was calculated. After incubation and detaching of fibroblast with 0.25% trypsin without EDTA, cells were distributed on the used siliconized tubes and centrifuged at 335 x g for 10 minutes then suspended in 2 mL 1 x phosphate buffered saline. Apoptosis was determined using Annexin V-FITC apoptosis with 2 fluorescent channels flowcytometry. After staining, cells were injected via ACEA flowcytometer and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively for each sample, 12,000 events were acquired and positive FITC and/or PI cells was quantified using ACEA NovoExpress™ software.

**Results:** According to Duncan multiple range tests, in necrotic phase, early apoptosis phase and late apoptosis phase, HBSS was highly significant to control and almond milk, while in normal intact cells phase almond milk showed the highest significant Q3 apoptotic cells against both control and HBSS. Moreover, HBSS also showed a significant to control, and almond milk as revealed by DMRTs.

**Conclusion:** Within the limitations of this study, it was concluded that almond milk has the ability to maintain viability of PDL cells, so may be considered as a viable option when other storage mediums as HBSS are not available at the site of accident.

**KEYWORDS:** Apoptosis, Almond milk, Cytotoxicity, HBSS, Fibroblast

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## INTRODUCTION

Avulsion of a tooth is the total dislodgment of the tooth from its socket secondary to traumatic injury, which results in great damage to the whole supporting structure surrounding a tooth which includes the periodontal ligament, cementum, alveolar bone, as well as the pulp tissue<sup>(1-4)</sup>. The most indicated treatment for tooth avulsion is reimplantation, which depends on reinserting the completely displaced tooth again in its socket<sup>(5,6)</sup>. Preservation of the viability of periodontal ligament cells (PDL) together with immediate tooth repositioning are crucial factors for a better prognosis of tooth reimplantation<sup>(7, 8)</sup>. In which, viable PDL cells was able to reattach through cell division, proliferation, and colonization on the root surface again to avoid tooth ankylosis and root resorption<sup>(7)</sup>. If the avulsed tooth cannot be repositioned within an hour, then the tooth should be preserved in an intermediate storing media to maintain the vitality of the surrounding ligament cells and allow cell proliferation<sup>(9)</sup>.

Many researchers had studied the properties of ideal storage media and concluded that the storage media should have an antimicrobial effect, not induce an antigenic reaction, root resorption or ankylosis, and be easily available and affordable<sup>(10-12)</sup>. However, the storage media with all these characteristics have not been identified yet<sup>(13, 14)</sup>. Tooth storage media have been classified into 4 groups according to their efficacy<sup>(15)</sup>. The first category, brilliant one: Hank's Balanced Salt Solution, Viaspan, natural milk, Propolis, and green tea. The second category, nearly good media: coconut water and egg white. The third group considered unfortunate media like contact lens solution and saline. The last group is non-favorable one as saliva and water. But, according to international organizations, HBSS and milk are considered the most powerful media for avulsed tooth<sup>(16)</sup>. Because it encompasses crucial metabolites with a balanced pH that permits conserving the viability of PDL cells up to two days<sup>(17, 18, 19)</sup>. However, the restricted availability to the laboratory uses together with its high cost make

it more mandatory to search for other available storage media with the same effect.

Almond milk is a phyto-genic-based creamy white solution attained from the clear blend of water mixed with ground amount of almonds which is considered as an alternative to cow's milk due to its multiple healthy nutrients, lactose-free, and low saturated fat<sup>(25)</sup>. It is a recommended storage medium that shows excellent characteristics in the matter of neutral pH, physiological osmolality, antibacterial activity, abundant growth factors and cell nutrients. It can preserve the viability of the periodontal ligament fibroblastic cells (PDLF) for 70- 90% up to 72 hours<sup>(20, 21, 22)</sup>. On contrary to HBSS, it is also inexpensive and commercially available<sup>(10,13,16)</sup>. From thorough search of relevant literature, there is few researches exploring the outcome of using almond milk as a storing medium on the periodontal ligament fibroblastic cells in comparison to HBSS using apoptosis assay for avulsed tooth maintenance.

## Methodology

The current study is double-blinded clinical trial Ethics approval no (678/2023), Faculty of Dentistry, Suez Canal University, Ismailia, Egypt.

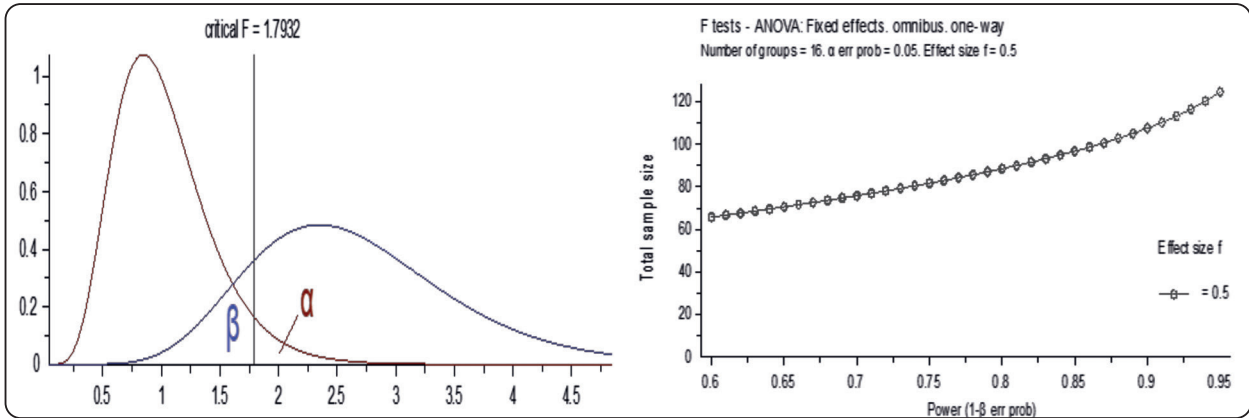
### i) Sample size calculation

To assess and evaluate cytotoxicity of different storage media (A, B) at different concentrations (0.01, 0.1, 1, 10, 100). A total sample size of 66 well were selected, in which different concentration for each media (a, b) was characterized by six wells and Group (C) was signified by 6 wells as shown in Tables (1) and (2).<sup>(26,30)</sup>.

$f$ : is the effect size

$$f = \frac{\sigma_{\mu}}{\sigma}$$

$$\sigma_{\mu}^2 = \frac{\sum_{i=1}^k n_j (\mu_i - \mu)^2}{N}$$



Design figure (1): Test graph of  $\alpha$ ,  $\beta$ , power (1- $\beta$ ) and total sample size.

TABLE (1): Tested Variables

Test Variable	Level	Denoted to
Type of storage media	A	Group (A), Hanks Balanced Salt Solution balanced salt solution.
	B	Group (B), Almond Milk almond milk.
	C	Group (C), Control fresh medium.
Concentration (mg/mL)	M <sub>1</sub>	0.01
	M <sub>2</sub>	0.1
	M <sub>3</sub>	1
	M <sub>4</sub>	10
	M <sub>5</sub>	100

TABLE (2): Factorial design for SRB assessment (n=6).

Variables	Concentrations (M)					Total	
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>		
Types of storage media	G-A	A M <sub>1</sub> (6 wells)	A M <sub>2</sub> (6 wells)	A M <sub>3</sub> (6 wells)	AM <sub>4</sub> (6 wells)	AM <sub>5</sub> (6 wells)	30
	G-B	BM <sub>1</sub> (6 wells)	B M <sub>2</sub> (6 wells)	BM <sub>3</sub> (6 wells)	BM <sub>4</sub> (6 wells)	BM <sub>5</sub> (6 wells)	30
	G-C	No medication (6 wells)					6
<b>Total</b>						66	

ii) Preparation and Grouping of materials

The tested storing media used for this study were HBSS, and almond milk. The two-storage media were primarily prepared as a 10 mg/mL stock. First, dilution with Dulbecco’s modified eagle medium (DMEM) to gain (0.01, 0.1, 1, 10, and 100 mg/mL).

The distribution of the experimental groups according to the medication was as follows:

**Group A:** hanks balanced salt solution.

**Group B:** almond milk.

**Group C:** (control group) fresh medium.

### iii) Cell culture

Human Gingival Fibroblastic cells attained from Nawah Scientific company., (Cairo, Egypt). Cells preserved in DMEM media with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO<sub>2</sub> atmosphere at 37°C.

### v) Routine Analysis IC50 Cytotoxicity assay

For detection of IC<sub>50</sub> of each storage media: samples were measured by Routine Analysis IC<sub>50</sub> (SRB) assay<sup>(31,32)</sup>. Sample of 100 µL cell suspension (5x10<sup>3</sup> cells) were placed in 96-well plates and incubated in media for 24 h. another aliquot of 100 µL media with drugs at various concentrations. After 72 h of drug exposure, media was replaced with 150 µL of 10% trichloroacetic acid (TCA) and were incubated at 4°C for 1 h. The TCA solution was erased, and washed 5 times by distilled water. Then, 150 µL of Tris-HCL (10 mM) were added to dissolve protein-bound SRB stain; the absorbance was calculated at 540 nm using a BMG LABTECH®- FLUOstar Omega microplate reader (Ortenberg, Germany).

### v) Flow cytometry test

This step was achieved in 500 µL siliconized tubes; each tube was involved 4 x 10<sup>6</sup> cells at the beginning of the procedure to recompense for cell loss.

Following sample incubation& detaching of fibroblast with 0.25% trypsin without EDTA, cells were distributed on the used siliconized tubes. Samples were centrifuged at 335 x g for 10 minutes and supernatant fluid was poured, then suspended in 2 mL 1 x phosphate buffered saline (PBS) (no calcium, no magnesium). Centrifugation and suspension were repeated three times. First suspension was in PBS and the following two times in 1 mL 1 x Annexin V binding buffer. The two different storage media were added to the tubes and incubated

at 37°C in humidified 95% air and 5% CO<sub>2</sub> for 24 h. Cells apoptosis and necrosis cell populations was determined using Annexin V-FITC apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK) coupled with 2 fluorescent channels flowcytometry. After treatment with test compounds for the specified duration, samples (10<sup>5</sup> cells) was collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Then, cells were incubated in dark with 0.5 ml of Annexin V-FITC/PI solution for 30 min in dark at room temperature based on manufacturer protocol. Following staining, cells were injected via ACEA Novocyte™ flowcytometer (ACEA Biosciences Inc., San Diego, CA, USA) and examined for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively ( $\lambda_{ex/em}$  488/530 nm for FITC and  $\lambda_{ex/em}$  535/617 nm for PI). For each sample, 12,000 events were acquired and positive FITC and/or PI cells was calculated by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA)<sup>(33-36)</sup>.

### vi) How to read the report

(Q1): Necrosis phase:

(Q2): Late apoptosis phase:

(Q3): Normal intact cells.

(Q4): Early apoptosis phase

## RESULTS

### Investigation IC50 Cytotoxicity assay

The viability (%) of the tested Hanks' balanced salts in concentrations from 0.01, 0.1, 1, 10, and 100 recorded an average ( $\pm$ SE) of 98.7 $\pm$ 0.99, 96.8 $\pm$ 0.99, 95.8 $\pm$ 0.69, 97.0 $\pm$ 0.74, and 98.3 $\pm$ 1.10; respectively. The statistical difference in viability was (p=0.042) which is significant .

Moreover, the viability (%) of the tested almond milk recorded an average ( $\pm$ SE) of 99.1 $\pm$ 1.42, 97.3 $\pm$ 1.14, 96.3 $\pm$ 0.77, 96.6 $\pm$ 0.84, and 94.8 $\pm$ 0.17;

respectively. The difference in viability was considered significant ( $p=0.042$ ) as revealed by ANOVA. According to two-way ANOVA, the overall corrected model was highly significant, concentration also induced a highly significant

( $p<0.001$ ) difference in viability (%). (Fig. 2) The relationship between concentrations and viability of HBSS and almond milk is represented by the regression trendline. (Fig.3, 4)

TABLE (3) Viability (%) of both HBSS and almond milk, tested in different concentrations from 0 to 100 (mg/mL).

Concentration	Groups						Independent t-test
	Hanks' Balanced salts			Almond Milk			
	Mean	SE	Tukey's	Mean	SE	Tukey's	
0	100.0	0.00	a	100.0	0.00	a	>0.05 ns
0.01	98.7	0.99	ab	99.1	1.42	ab	0.802 ns
0.1	96.8	0.99	ab	97.3	1.14	ab	0.771 ns
1	95.8	0.69	ab	96.3	0.77	ab	0.307 ns
10	97.0	0.74	ab	96.6	0.84	ab	0.385 ns
100	98.3	1.10	ab	94.8	0.17	b	0.032 *
ANOVA (p-value)	0.042*			0.012*			
Correlation	p= 0.190 ns			r= -0.801; p=0.014*			
Regression R2	0.0145; p=0.190 ns			0.333; p=0.014*			
Two Way ANOVA							
Corr. Model	0.003**						
Concentration	<0.001***						
Group	0.403 ns						
Group x conc.	0.166						

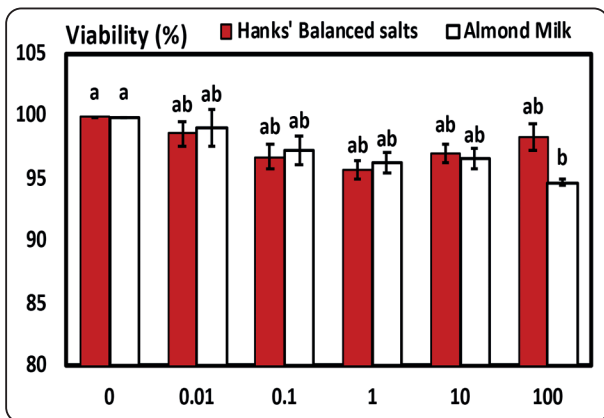


Fig. (2) Bar chart representing the viability (%) of both HBSS and almond milk, tested in different concentrations from 0 to 100 (mg/mL).

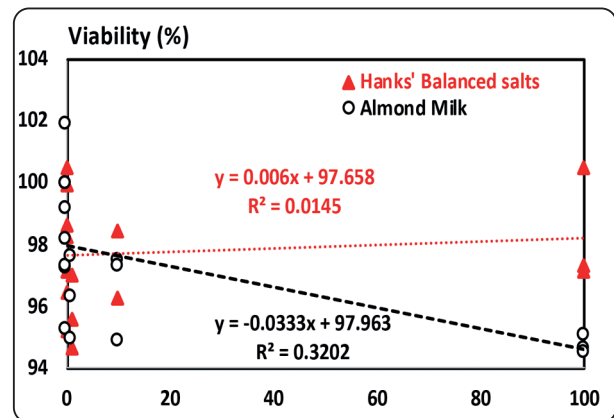


Fig. (3) Regression trendline showing the relationship between concentrations and viability of HBSS. and almond milk.

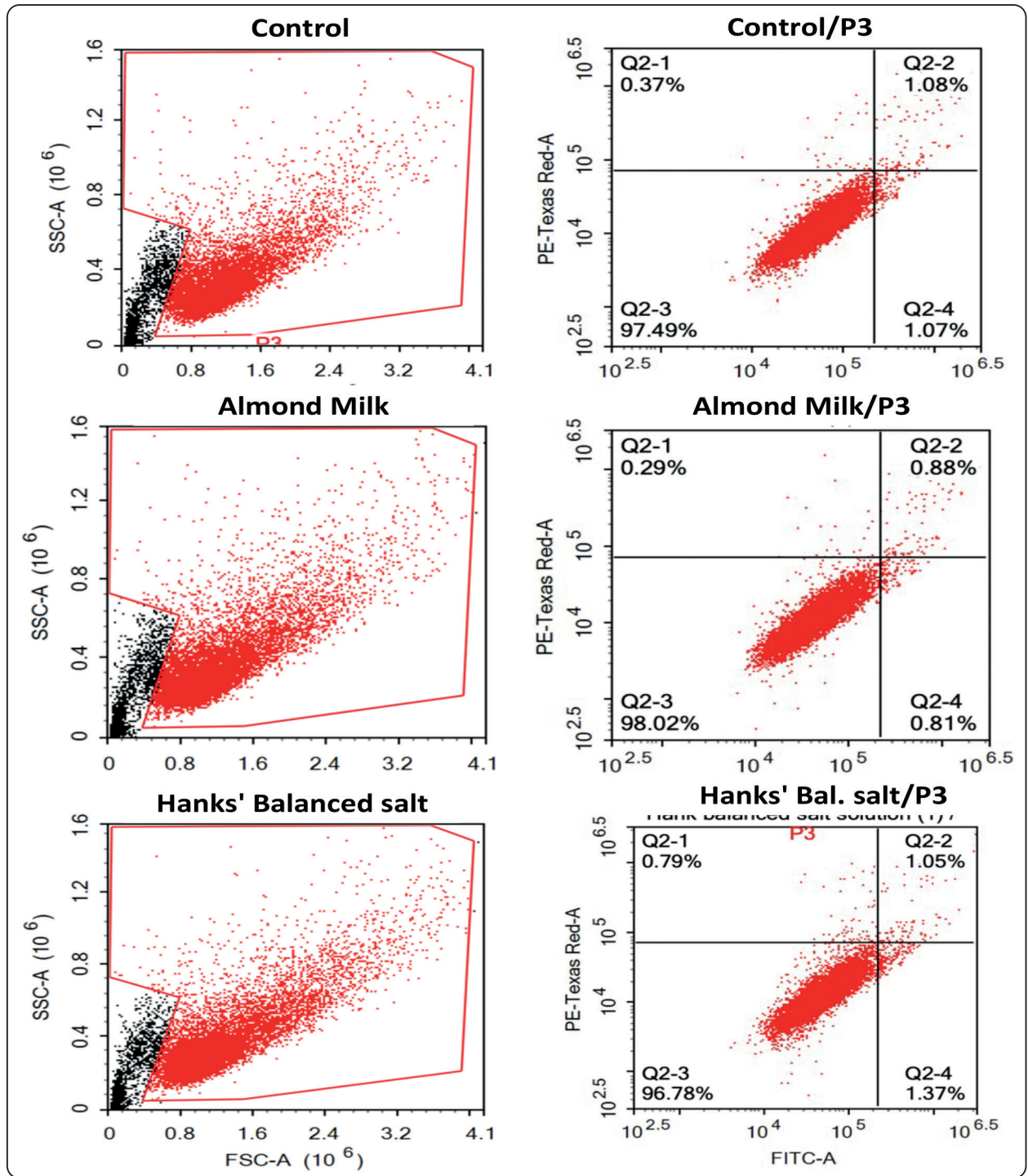


Fig. (4) Cytometric profiles of three studied groups, control, almond milk, and Hanks' balanced salts.

**The cell viability assay**

Data represent levels of gingival fibroblast viability, following tooth avulsion and storage media exposure relatively to the DMEM control was described per quarter as follows (Table 3)

Q1: The cellular count recorded an average ( $\pm$ SD) of 42.0 $\pm$ 9.54, 40.0 $\pm$ 11.27, and 72.3 $\pm$ 8.33 in control, almond milk and HBSS, respectively. The change between the three groups was significant. Accordingly, HBSS was highly significant to control and almond milk, however, no significant difference between control and almond milk as showed with Duncan multiple range tests (DMRTs). (Fig.5a)

Q2: The apoptotic cell records an average ( $\pm$ SD) of 98.3 $\pm$ 14.64, 80.7 $\pm$ 8.74, and 109.3 $\pm$ 8.33 in control, almond milk and HBSS, respectively. Alteration among the three groups was significant with p=0.048, Accordingly, HBSS showed the highest significant Q2 apoptotic cells against control and almond milk, however, no significant difference

between control, and almond milk as revealed by DMRTs. (Fig.5b)

Q3: The apoptotic cell count recorded an average ( $\pm$ SD) of 8,702.3 $\pm$ 34.79, 9,217.0 $\pm$ 9.54, and 9,186.3 $\pm$ 10.97 in control, almond milk and HBSS, individually. The statistical evaluation among the tested groups was significant. Accordingly, almond milk showed the highest significant Q3 apoptotic cells against both control and HBSS. Moreover, HBSS also showed a significant difference to control, and almond milk as revealed by DMRTs. (Fig.5c)

Q4: The apoptotic cell count recorded an average ( $\pm$ SD) of 101.7 $\pm$ 7.64, 84.0 $\pm$ 6.93, and 125.7 $\pm$ 4.04 in control, almond milk and HBSS. The statistical evaluation among the tested groups was significant. Accordingly, HBSS showed the highest significant Q4 apoptotic cells against control and almond milk, however, no significant difference between control and almond milk as revealed by DMRTs. (Fig.5d)

TABLE (4) Record of Q1, Q2, Q3, and Q4. Mean tracked by letters vertically or horizontally are significantly different according to DMRTs.

Group	Apoptosis												ANOVA RM
	Q1			Q2			Q3			Q4			
	Mean	SD	DMRT	Mean	SD	DMRT	Mean	SD	DMRT	Mean	SD	DMRT	
Control	42.0	9.5	h	98.3	14.6	ef	8702.3	34.8	c	101.7	7.6	ef	<0.001***
Almond Milk	40.0	11.3	h	80.7	8.7	fg	9217.0	9.5	a	84.0	6.9	fg	<0.001***
Hanks' BS	72.3	8.3	g	109.3	8.3	de	9186.3	11.0	b	125.7	4.0	d	<0.001***
ANOVA	0.012*			0.048*			<0.001***			<0.001***			
Two way ANOVA													
Group	<0.001***												
Q	<0.001***												
Group x Q	<0.001***												

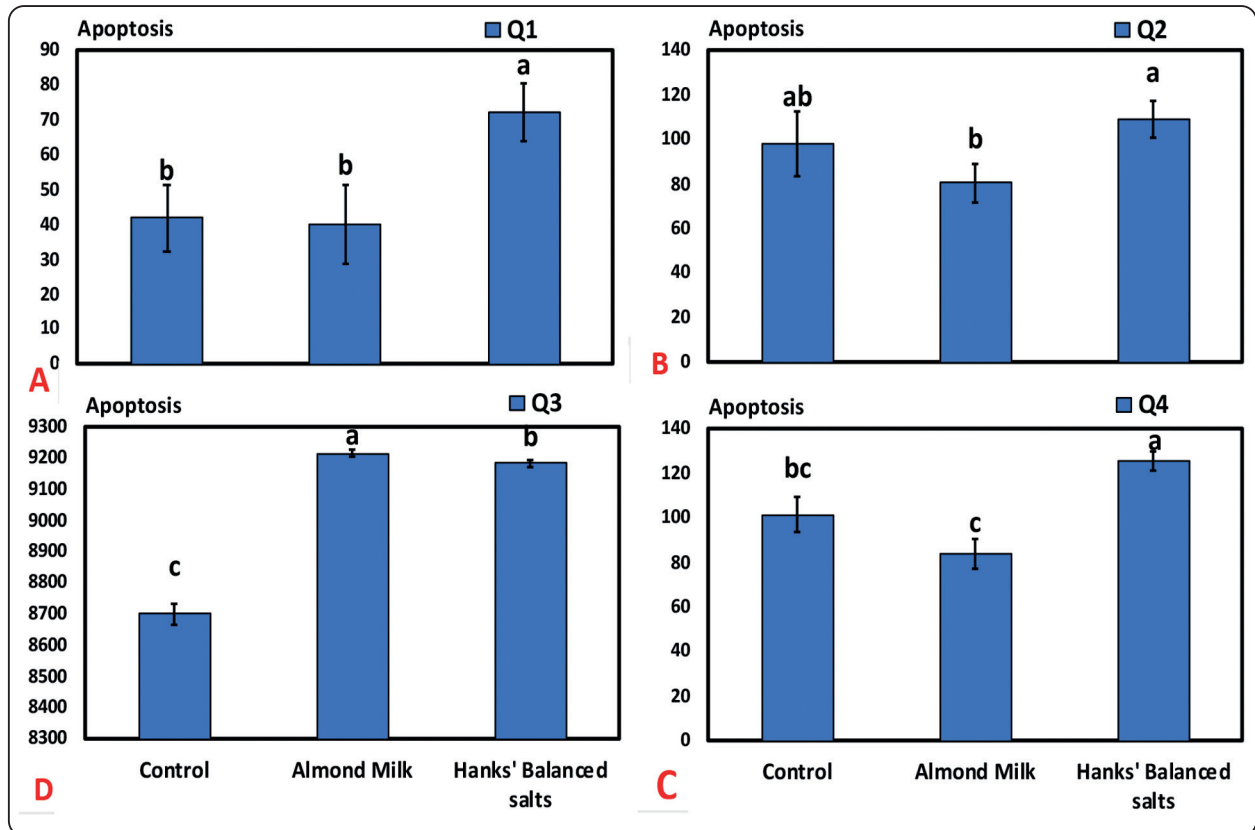


Fig. (5) Apoptosis is presented as mean and standard deviation in Q1, Q2, Q3, and Q4.

### DISCUSSION

Avulsion of permanent tooth is considered the most serious traumatic injury resulting in aesthetic, functional and psychological problems to injured patient (37, 38). The recommended treatment in such case is instant replantation of the dislodged tooth; however, it is not always possible to do so. Viability of the PDLFs on the root surface affect prognosis. So, using favorable storage media is a crucial point (39- 41).

HBSS is the widely recommended storage media, as it preserves the components of periodontal ligament tissue in its normal physiological conditions (42, 43). However, it is expensive and not always available at the site of accident (44). Hence, the need for other natural storage media which are easily available and affordable is in the search.

Almond is a widespread characterized by some properties that makes it a good choice for tooth preservation; as it is slightly acidic, which makes it able to neutralize any harmful bacteria that may present on tooth surface, and has anti-oxidative and anti-inflammatory properties (45). Additionally, almond comprises several healthy nutrients as calcium and vitamin D which are both important for tooth health (46).

The ability of numerous types of storing media in preserving viability of PDLF in vitro was detected by cultured PDL cells or by direct immersion of the avulsed tooth in the test media and subsequent counting of the number of viable cells (47, 48). Here, we compared the ability of HBSS and almond milk on maintaining the viability of the cultured HGF using apoptosis/necrosis assay. Therefore, vital, early and late apoptotic and necrotic cells can be



distinguished and analyzed by microscopic flow-cytometry<sup>(49)</sup>. Due to their quick and easily growth Human gingival fibroblasts were used<sup>(50)</sup> periodontal ligament availability, which allows determining the effect of the storage media on its viability<sup>(51)</sup>.

Regarding the results of this study, the viability of PDLF cells immersed in almond milk was comparable with that of control group; which comes in constant with the study of **Oikarinen et al.**<sup>(52)</sup> showing that both milk and control group didn't alter the proliferation of PDLF. In contrast, **Sinpreechanon et al.**<sup>(53)</sup> have established that PDLF cell survival stored in almond milk was slightly lower than that stored in control group. These changeability in the outcomes may be due to different sources and ingredients of the tested milk.

Also, it was noted that the viability of PDLF cells placed in almond milk group during all stages of apoptosis/necrosis assay in this study was statistically significant than that of the HBSS group. These conclusions are in harmony with earlier studies display capability of milk than HBSS in maintaining viability of cells<sup>(54-57)</sup>; as almond milk contains sugars, proteins, and antibacterial agents that can enhance proliferation of PDLF cells on reimplantation of knocked out tooth. Nevertheless, in other experiments, effectiveness of milk is inferior to<sup>(58,59)</sup> or close to HBSS<sup>(60-63)</sup>.

## CONCLUSION

While there is limited research on the ability of almond milk in preserving avulsed teeth and maintaining viability of PDL cells, it may be considered as a viable option when other storage mediums as HBSS are not available at the site of accident.

## Limitations

1- The study was conducted on PDLF cells from extracted teeth and not on an avulsed tooth of live patients. Therefore, it's difficult

to determine whether almond milk would be effective in preserving a live tooth as it was in preserving the PDLF cells of extracted teeth used in the study.

2- It's important to note that extra-oral time is one of the important factors that should be considered on preserving an avulsed tooth. The longer the tooth is out of the socket, the lower its chances of survival. Therefore, this study can't determine whether almond milk would be able to preserve the viability of PDLF if stored in it more than one hour or not.

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