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Evaluation of the Effect of Green Tea Extracts on Periodontal Ligament Fibroblasts Viability versus Hank's Balanced Salt Solution

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ABSTRACT

Purpose: To assess the effect of Green Tea Extract (GTE) on the periodontal ligament fibroblasts viability comparing to Hank's Balanced Salt Solution (HBSS). **Materials and methods:** Periodontal ligament fibroblasts were obtained from 30 freshly extracted sound premolars and cultured in Dulbecco's Modified Eagle Medium (DMEM). Cell viability was determined by storing the cells in Hank's balanced salt solution (Group I), Green tea extract (Group II) and fresh Dulbecco's Modified Eagle Medium (DMEM) (Group III) as positive control, for 1,3,6,12,24 and 48h at 37°C using MTT assay (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). **Results:** Statistical analysis was performed by one-way analysis of variance (ANOVA) and post hoc tests. The findings of this study revealed that there was no significant difference between groups at 1h and 3h time intervals, while GTE showed considerably better protective effect than HBSS at 6 hrs. (p = 0.002), at 12 hrs. (p = 0.001), 24 hrs. (p = 3.38E-03) and 48 hrs. (p = 1.65E-03). **Conclusion:** Within the boundaries of the present study, it showed that GTE had higher protective effect on periodontal ligament fibroblasts than HBSS and hence can be used as alternative medium to HBSS.

KEYWORDS

Avulsion, Green Tea Extract, cell viability, Hank's Balanced Salt Solution.

INTRODUCTION

Tooth avulsion or complete displacement of the tooth from the socket causes practical, psychological and aesthetic issues as well as, impaired neurovascular supply, periodontal ligament cell loss, pulp necrosis and infection [(1)].

- Paper extracted from Master thesis titled "Evaluation of the Effect of Green Tea Extracts on Periodontal Ligament Fibroblasts Viability versus Hank's Balanced Salt Solution"

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Because of their anatomic position in the oral cavity, the maxillary central and lateral incisors are the top to get fractured. The most common etiologic factor for tooth fracture is falls accounting for up to 31 to 64%. Comes next, sports injuries (40%), cycling accidents (19 %), traffic accidents (8 %), and physical violence (7 %). Commonly, single tooth fracture is noted, but sports injuries, traffic accidents, and physical violence may cause fractures of multiple teeth [(2)].

The perfect scenario is immediate replantation of the tooth, however, sometimes it's hard due to several situations as faraway accommodation from dentist or accompanied injuries and hence correct storing of teeth is mandatory [(3)].

The prognosis of an avulsed tooth depends considerably on extra-oral time span and the storage medium where the avulsed tooth is retained prior to replantation [(4)]. The prognosis of the replanted teeth is directly proportional to the viability of the periodontal ligament cells which is highly dependent on the storage medium preserving them. Storage media with suitable pH, osmolality and nutritional substances are widely accepted as ideal, yet there is no accord on the best medium to secure cell survival and avoid ankylosis and replacement resorption [(5)].

Several media have been recommended for avulsed teeth [(6)]. In the midst of experimented solutions, HBSS has been warranted to protect the viability, cell proliferation, and clonogenic capacity of periodontal ligament cells [(5)].

In order to help cell survival, HBSS has important characteristics, in that it is biocompatible, highly nutritious, and has an adequate pH balance and osmolality. Its restricted accessibility, however, has found it unserviceable [(7)].

As a possible storage medium, GTE is currently suggested. Green tea (GT), derived from Camellia sinensis, is a widely consumed drink after water worldwide. GTE includes the largest group of polyphenols including catechin, epicatechin, gallate epicatechin, epigallocatechin, and 3-gallate epigallocatechin (EGCG). Such polyphenols have anti-inflammatory and antioxidant effects. [(8)].

Therefore, the effect of GTE vs. HBSS on periodontal cells viability was evaluated using MTT assay (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide).

MATERIALS AND METHODS

Case selection:

Human PDL cells were derived from thirty sound premolars extracted for orthodontic aims, cultured in Dulbecco's Modified Eagle Medium (DMEM). Ethical approval for the use of extracted human teeth was obtained in compliance with guidelines from research ethics committee approval of dental medicine faculty - Al-Azhar University (Girl's branch), Cairo, Egypt.

The research removed teeth with periodontal involvement, enamel defects (such as amelogenesis imperfecta, dentinogenesis imperfecta, enamel hypoplasia, etc.) and those acquired from medically comprimised patients [(9)].

Collection of teeth:

After the thirty clinically healthy premolars were extracted, teeth were held by forceps from the coronal section then put under running water without scraping the root to remove the soft debris from the tooth surface. From the roots of these teeth, human PDL cells were derived and then preserved until use in DMEM.

Grouping of teeth:

Cultured Cells from thirty teeth were divided into 3 groups in six 96-well plate:

Group I: Hank's Balanced Salt Solution (HBSS).

Group II: Green Tea Extract (GTE).

Group III: Dulbecco's Modified Eagle Medium (DMEM) (positive control).

Preparation of Green Tea Extract (GTE):

10 g of GT leaves were immersed in 100 mL of boiling distilled water for 5 minutes and filter sterilized [10].

Preparation of cell culture [8]:

- Fibroblasts were disaggregated enzymatically using collagenase type IV and Trypsin/EDTA. In a 6-well plate (fig.1A), the cells were cultured in DMEM and kept in incubator at 37 °C in a humidified atmosphere containing 5% CO_2 until confluence (fig. 1B). Every three days, the media was exchanged by fresh one.
- After reaching confluence, dispersing of the attached cells was done using trypsin/EDTA. Under inverted microscope, cells were checked for dispersing.
- In a 96-well tissue culture plate (fig.1C), in each well, 5-6 × 10^3 cells were seeded and incubated. Cells from 3-5 passages were used.
- 48 hours later, the media was removed and 100μl of Dulbecco's eagle medium, hank's balanced salt solution and green tea extract was added for the required time interval 1, 3, 6, 12, 24 and 48 h in the incubator. Cells stored in Dulbecco's eagle medium were used as positive control.
- After the required time intervals, storage media were removed and cells were rinsed 3 times with phosphate buffered saline (PBS). Into each well, 50 μl of MTT solution (MTT, Sigma) was added (fig. 1D). Plates were then incubated at 37 °C.
- 4 hours later, the resulting formazan crystals were dissolved by adding 100μl of dimethyl sulfoxide (DMSO) (MTT solubilization solution).
- Gentle mixing in the gyratory shaker was done to enhance dissolution.
- Cell viability was measured through the differential optical densities at 450 nm and 630 nm as reference wavelength by a spectrophotometer. The cells stored in DMEM were used as a positive control for cell growth.

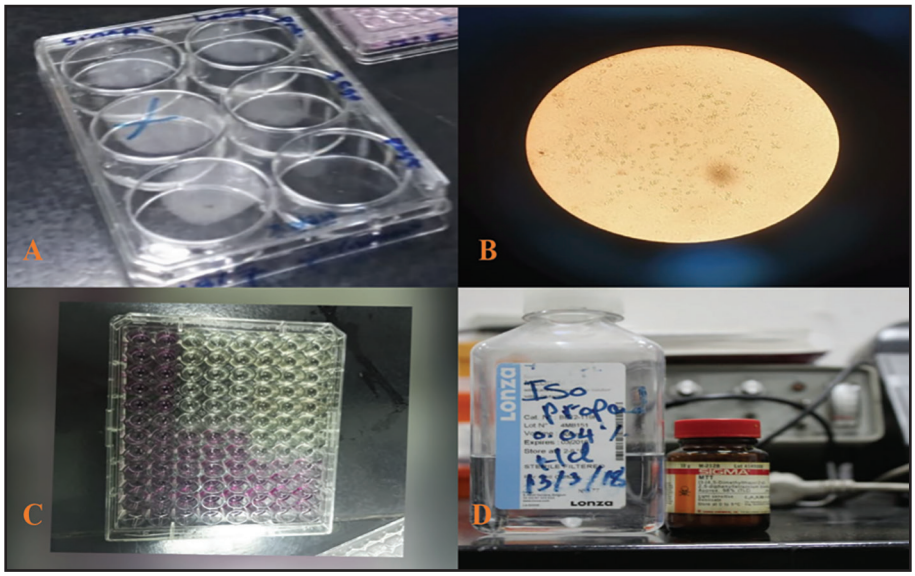

Figure 1: A) 6-well plate. B) 75%-80% cellular confluence. C) 96-well plate. D) In vitro Toxicology Assay Kit MTT based

RESULTS

Statistical analysis was done using One-way analysis of variance (ANOVA) test where the significance level was set at p ≤0.05. Then Tukey's Post Hoc test was used to find out which group these disparities were in favor of.

From the ANOVA test it was concluded that were no statistical difference between groups at 1h and 3h intervals; P=0.139 and P=0.273 respectively, while significant differences between groups began to appear after 6 hours, and these differences increased with time (Table 1).

From Tuckey's post hoc test the following was concluded:

After 1 hr.*:** Viability of cells in HBSS media was higher than others with slight differences that ***were not statistically significant (P > 0.05).

After 3 hrs.* :** The viability of cells in GTE media was higher than others with slight differences that ***were not statistically significant (P > 0.05).

***After 6 hrs.* :**

- There were statistically significant differences between the DMEM and HBSS groups in favor of the DMEM group which had the highest mean of viability.
- There were statistically significant differences between the GTE and HBSS groups in favor of the GTE group with the highest mean of viability.
- There was no statistically significant differences between DMEM and GTE after 6 hours, P-value = 0.08.
- The HBSS group had a lower mean of viability than the GTE and DMEM groups.
- The lowest P-value was achieved between groups DMEM and HBSS, which indicated the highest differences in means between the two groups.

Post time 12 to 48 hours:

- There were statistically significant differences between the mean of the DMEM group, and means of GTE and HBSS groups in favor of the DMEM which has the highest mean of viability.
- There were statistically significant differences between the GTE and HBSS groups in favor of the GTE group with the highest mean of viability.
- The HBSS group had a lower mean of viability than the GTE and DMEM groups.
- The lowest P-value was achieved between groups DMEM and HBSS, which indicated the highest differences in means between the two groups. (Table 1, 2, 3, fig. 2).

Table (1) *Descriptive statistics and results of one way ANOVA:*

Time	DMEM		GTE		HBSS		P-value
	Mean	SD	Mean	SD	Mean	SD	
Post 1 hr.	0.418	0.001	0.418	0.003	0.422	0.002	0.139
Post 3 hr.	0.435	0.004	0.437	0.006	0.428	0.008	0.273
Post 6 hr.	0.445	0.007	0.428	0.011	0.387	0.003	2.47E-04*
Post 12 hr.	0.458	0.005	0.420	0.016	0.354	0.008	5.67E-05*
Post 24 hr.	0.498	0.011	0.333	0.015	0.282	0.008	9.96E-07*
Post 48 hr.	0.565	0.005	0.250	0.010	0.214	0.005	2.31E-09*

** Significant at P ≤ 0.05.*

Table (2) *Post Hoc Tests (Tukey's) post 6-48 hours:*

Dependent variable	(I) Group	(J) Group	Mean difference (I-J)	Std. error	P-value
Post 6 hrs.	DMEM	GTE	0.017200	0.0064	0.080
		HBSS	.058767*	0.0064	2.28E-04
	GTE	DMEM	-0.017200	0.0064	0.080
		HBSS	.041567*	0.0064	0.002
	HBSS	DMEM	-.058767*	0.0064	2.28E-04
		GTE	-.041567*	0.0064	0.002
Post 12 hrs.	DMEM	GTE	.038200*	0.0086	0.010
		HBSS	.103767*	0.0086	4.75E-05
	GTE	DMEM	-.038200*	0.0086	0.010
		HBSS	.065567*	0.0086	0.001
	HBSS	DMEM	-.103767*	0.0086	4.75E-05
		GTE	-.065567*	0.0086	0.001
Post 24 hrs.	DMEM	GTE	.164333*	0.0092	5.17E-06
		HBSS	.216000*	0.0092	1.15E-06
	GTE	DMEM	-.164333*	0.0092	5.17E-06
		HBSS	.051667*	0.0092	3.38E-03
	HBSS	DMEM	-.216000*	0.0092	1.15E-06
		GTE	-.051667*	0.0092	3.38E-03
Post 48 hrs.	DMEM	GTE	.314467*	0.0057	2.49E-07
		HBSS	.351233*	0.0057	2.49E-07
	GTE	DMEM	-.314467*	0.0057	2.49E-07
		HBSS	.036767*	0.0057	1.65E-03
	HBSS	DMEM	-.351233*	0.0057	2.49E-07
		GTE	-.036767*	0.0057	1.65E-03

The mean difference is significant at P≤0.05 level (*)

The highest differences in means between two groups (*)

(I) and (J) are just letters to differentiate between the columns and discuss the direction of comparison in column (Mean Difference).

Table (3) *Percentage of periodontal cells viability:*

Sample code	1h	3h	6h	12h	24h	48h
PDL cells/ GTE	99.84 %	100.37 %	96.15 %	91.64 %	67.01 %	44.33 %
PDL cells/ HBSS	100.85 %	98.39 %	86.82 %	77.34 %	56.63 %	37.82 %

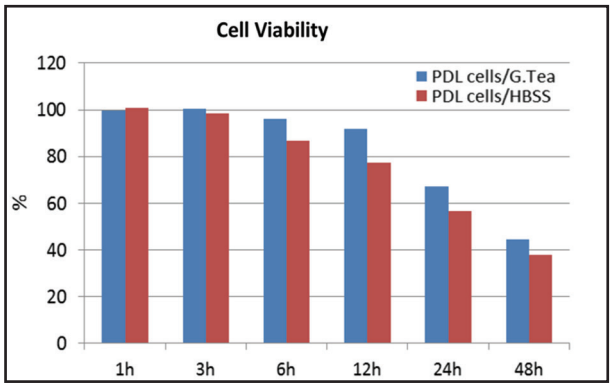

Figure 2 Bar chart showing the percentage of periodontal cells viability in both groups

DISCUSSION

Children are highly subjected to accidents during playing and the most common is avulsion to their front teeth. Placing the avulsed tooth in an adequate medium in case of failure of immediate replantation is of great importance to save the vitality of periodontal ligament cells and hence replantation can be done by the dentist [11]. The chief result of this study was the verification of GTE as a storage media being equal or even better than HBSS.

In the present analysis, HBSS was used because it is the most studied avulsed tooth storage solution, and several authors have considered it a gold standard solution. HBSS is a pH-balanced salt solution that contains important cell maintenance metabolites and glucose [12].

However, accidents may occur in schools, homes and sporting clubs, where HBSS is not accessible[6]. The HBSS is also expensive and unavailable [13]. GTE has been used because it is an available drink that is consumed globally and contains large levels of valuable nutrients including antioxidant polyphenols [8].

Cell viability was assessed using the MTT tetrazolium method in the present analysis. Due to its key advantages of speed, accuracy and not requiring radioisotope use. It is also a valuable method to determine the toxicity of recently developed drugs [14].

In this study, the results showed that there was no significant difference statistically between GTE and HBSS in maintaining the periodontal fibroblasts viability at 1 and 3h time intervals. Significant difference appeared after 6, 12, 24 and 48 h, when GTE showed higher preserving effect; 96.15 %, 91.64 %, 67.01 % and 44.33 % respectively, and HBSS; 86.2 %, 77.34 %, 56.63 % and 37.82 % respectively.

The present study came in harmony with another in vitro study that aimed at determining the potential of GTE in preserving the viability of PDLFs compared with seven different storage media; DMEM, tap water, Hank's balanced salt solution (HBSS), whole milk, hypotonic sucrose solution, GTE, and GTE + sucrose for 1, 2, 4, and 24 h at 37°C using same assessment method, tetrazolium salt-based colorimetric (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay. It showed that GTE had considerably higher protective impact than HBSS despite it using different GTE preparation method. The 10 gm of GT leaves in 100 ml of water in the previous study, the water temperature was increased moderately till 80°C and kept in this temperature for 45 minutes, while in the present study, the GT leaves were boiled for 5 minutes [8]. This may be due the anti-oxidative polyphenols and protective nutrients of the GTE.

Meanwhile, it didn't harmonize with another study that was done to evaluate the potential of GTE in periodontal ligament cells preservation in comparison to water (negative control), and Hank's balanced salt solution (HBSS) (positive control), for 1, 3, and 15 hours at 4 °C, using the trypan blue exclusion technique, that showed no difference between HBSS and GTE at all-time intervals, while in this study, there was a significant difference between them after 6 hours till 48 hours in favor of GTE. This difference may be because of using different temperature (4°C) of all the storage media during all time intervals [15]. Whilst in this study the media were kept in room temperature (37 °C) which is similar to the real circumstances.

CONCLUSION

Hence in the light of the present study and within its parametric limits, GTE showed a similar effect to HBSS and even better effect at longer time intervals. GTE can be used as another affordable substitute storage media for the avulsed tooth.

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