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Egg White-Milk Mixture as a Novel Biological Storage Media for Avulsed teeth Periodontal Ligament Cells: An *In-Vitro* Study

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Abstract

Purpose: This study aimed to evaluate egg white-milk mixture as a novel storage medium for avulsed teeth compared to Hank's Balanced Salt Solution (HBSS) media.

Materials and media: Ninety incisors teeth of 23 New Zealand rabbits were atraumatically extracted and divided into 6 equal groups. Group 1, 2 & 3 were immediately stored in HBSS media for 1, 4 & 6h respectively, while group 4, 5 & 6 were stored in egg white-milk for 1, 4 & 6h respectively. The teeth were prepared for histological investigation and digital counting of periodontal ligament (PDL) cells using trypan blue exclusion technique.

Results: Teeth stored in HBSS showed that 65.75%, 48.25% & 38.08% of PDL cells were vital after 1, 4 & 6h storage time respectively, while egg white-milk reported 49%, 50.06% & 47.67% for the same storage times. In comparing, there is highly significant difference in favour of HBSS after1h storage time. However, this difference is converted in favour of the novel mixture with insignificant difference after 4h and high significant difference after 6h.

Conclusion: Egg white-Milk mixture has the ability of replenishment of metabolites in depleted cells. Moreover it has significant superior properties than the approved evidences of HBSS especially for teeth stored for more than 4h.

Keywords: Avulsed teeth; Storage media; Periodontal ligament cell

Introduction

Avulsion is one of the most complex traumatic injury affecting children teeth [1].Tooth avulsion is complete tooth displacement out of its socket [2,3]. It accounts for 0.5-16% of traumatic injuries in the permanent dentition and 7-21% in the primary dentition. It may occur at any age and creates special problems for the patient and the dentist [3,4]. Avulsion injuries represent a potential threat for affected teeth periodontium and pulp so it requires quick emergency intervention. The cells of the pulp and periodontal ligament (PDL) begin to deteriorate due to cutting of Blood supply, lack of humidity, and environmental factors (e.g. bacterial contamination). Avulsed tooth Management includes management of the pulp and PDL cells, with the latter being far more important [5-8].

Tooth replantation prognosis is largely dependent on the vitality of the PDL cells at replantation time [2]. The key of success is immediate replantation or maintenance of the avulsed tooth in a compatible storage medium to keep these cells survival before replantation into the socket [9]. Replantation of a tooth within 5 min usually ensures prompt return of the PDL differentiation and stem cells to normal function [10]. However, more than 15 min of dry storage, PDL stem cells are no longer able to differentiate into fibroblasts [11]. Furthermore, after 30 min all the remaining PDL and stem cells on the tooth root are likely to become necrotic [12,13]. Immediate replantation results in PDL healing up to 85% of mature teeth [14]. Other contributing factors like knowledge of the appropriate actions to take and proximity of the scene to a dentist or dental clinic should be considered [15].

Many storage media were used as HBSS, artificial saliva and saline. HBSS have the best PH value and osmolality that are more important than the chemical composition. Moreover it is non-toxic, biocompatible, contain glucose, magnesium and calcium required for cell survival, does not require refrigeration and have long shelf life-2 years.So it is considered the ideal storage medium preserving vitality, mitogenicity and clonogenic capacity of PDL stem cells of avulsed teeth [1,7,16]. However, it is expensive, not easily available especially at trauma sites and poor populations [6,17].

Natural storage media like Milk, Egg albumin, Propolis, Green Tea Extract, soymilk and Coconut water were suggested as storage media and may rescue the avulsed tooth. They were promising concerning the organization of collagen fibres and the number of viable cells. Some suggest that they can be perfect media for storing avulsed teeth but they need additional studies [4,6,18-21]. Some considered milk as a gold standard for transporting avulsed teeth. It is a physiological medium, which contains growth factors and nutrients, Small bacterial contents, isotonic, physiological pH and osmolality [22,23]. While others reported superior results and properties with egg white than milk since it is also contains low microbial contamination, nutrients and water [1,4]. Each egg white nearly contains 4.7 grams of 40 different proteins, 0.3 grams of carbohydrate, 62 milligrams of sodium, and the remainder being water [23].

HBSS, milk and egg white were widely investigated and showed obvious contradicted results. Others reported that HBSS showed insignificant higher viable PDL cells after 1 and 3h storage time. While milk came second after 1h and third next to egg white after 3h storage time with insignificant values [24]. However, other studies demonstrated that egg white reported higher viable PDL cells than milk after half hour storage time [25,26]. On the same context Khademi., et al. found that teeth stored in egg white for 6 to 10h had a better incidence of repair and lower surface resorption than those stored in milk [20]. According to the International Association of Dental Traumatology and the American Academy of Pediatric Dentistry milk is considered as the second or third best transportation media for avulsed teeth include after Viaspan and/or HBSS [23,27].

Further studies are required to interpret theses adverse results, as there are wide variations in milk and egg white composition and quality. Also there is no study concerned the evaluation of egg white-milk mixture as storage media that could have a synergistic action on cellular viability. So the propose of this study was to evaluate the efficacy of egg white-milk mixture in maintaining the viability of PDL compared to HBSS.

Methods

The study was previously submitted and approved by the Ethical Committee of the National Research Centre.

Samples selection

Twenty three healthy New Zealand rabbits (older than 5m age) were selected. All rabbits were born and living in Animal Reproduction Research Institute (ARRI) and housed in cages under controlled environment with free access to food and water.

Sample preparation

All rabbits were anaesthetized according to Cornell University Institutional Animal Care and Use Committee rabbit anaesthesia protocol [28]. Using xylazine (Xyla-Ject injectable sol. ADWIA Co. S.A.E Egypt) 1-5mg/Kg IM which gave 30-60 minutes working time. Atraumatic extraction (using a Periotome) of rabbit's incisors teeth (2 upper and 2 lower) was done to simulate the clinical scenario of dental trauma and avulsion [19].

Teeth classifications and grouping

The extracted teeth (90) were equally divided and stored immediately post-extraction in HBSS media and egg-white-milk mixture media [20,24]. These teeth were sub-classified according storage media and time of storage into 6 equal groups (each group was formed of 15 teeth) as following:

Group 1, 2 & 3: Teeth were stored in HBSS media for 1, 4 & 6h respectively

Group 4, 5 & 6: Teeth were stored in egg albumin-milk mixture media for 1, 4 & 6h respectively.

Materials and media

Egg white

Egg albumin is composed mainly of 76.15% water, 12.56% proteins, 0.72% carbohydrates, 9.51% total fats, minerals (Ca, Fe, Mg, Ph., Po, Na and Zn), vitamins (A, C, B complex & E) and sugars. The pH of egg albumin is about 6.6-7 and its osmolality is about 251-298 mOsm/kg [4,29].

Milk

Milk is composed of Water (88.18) g, Protein (1.75 g), Lipid (3.41 g) and Carbohydrate (6.16g). Also, it contains many Minerals (Ca mg 33, Fe mg 0.39, Mg mg 6, P mg 74, K mg 114, Na mg 78 & Zn mg 1.18) and Vitamins (Thiamine mg 0.012 & Riboflavin mg 0.088). The pH of milk is about (6.5-7) and its osmolality is about (290-375 mOsm/kg) [4,18,30].

Hank's Balanced Salt Solution

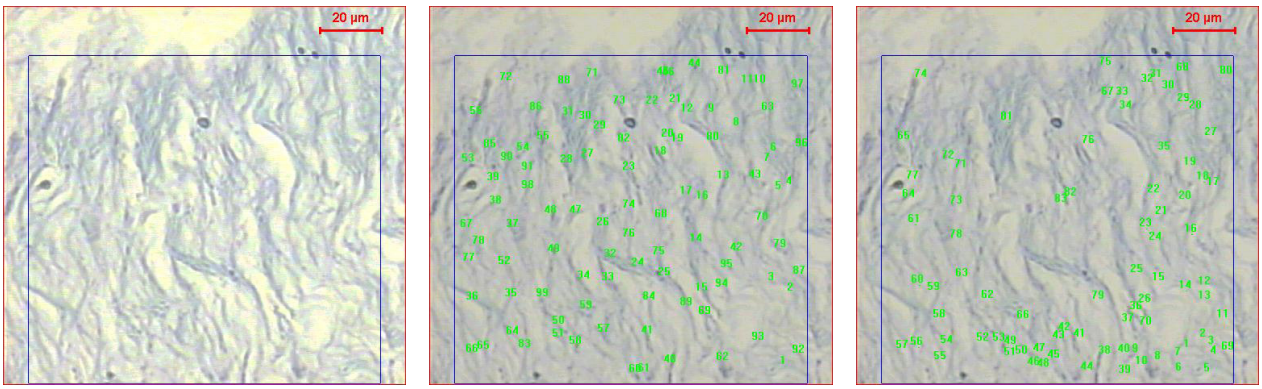
HBSS (Save-A-ToothTM, Sigma- Aldrich Inc., Pottstown, PA, USA.) composed of 8 g/L sodium chloride; 0.4 g/L of D-glucose; 0.4 g/L potassium chloride; 0.35 g/L sodium bicarbonate; 0.09 g/L sodium phosphate; 0,14 g/L potassium phosphate; 0.14 g/L calcium; Chloride; 0.1 g/L magnesium chloride and 0.1 g/L magnesium sulphate. Moreover, it has neutral pH (7.2) and osmolality (320 mOsm/kg) [30,31].

Eggwhite and milk mixture**

Mixture of milk with egg white in a ratio of 1:1by volume. The resulted pH of the new mixture was 6.6-7.8 as measured by pH meter. All teeth were stored at room temperature 27 °C and prepared for histological investigation.

Histological examination

By the end of storage time teeth crowns were grasped atraumatically with holder and washed with normal saline. Care was taken to avoid periodontal ligaments harm. Teeth were immersed into 10% formalin for fixation. Then the extracted teeth PDL were removed from roots using sharp curette 3mm apical to Cemento-Enamel Junction to exclude damaged PDL cells from the extraction forceps and the junctional epithelial cells. These PDL fibres were stained with Trypan Blue using Trypan Blue Exclusion Technique. The stained periodontal ligaments were histologically examined. The viable and non-viable cells were counted using digital microscope with magnification 20X with a corresponding special software in area 121 x 129 Mm for each slide in General Pathology Lab. of National Research Centre (Figure 1) [19-21].



A. Original section B. Non vital cells C. Vital cells

Figure 1: Trypan Blue stained histological section of tooth periodontal ligaments showing of the vital and dead cells

Statistical analysis

Data were collected and analysed using SPSS.16 statistical software (IBM Corp., Armonk, N.Y., USA). Data were analysed by one-way analysis of variance (ANOVA), post hoc Tukey and paired t-test at significance level of $P < 0.05$.

Results

Histological investigation of PDL cells of teeth immersed in HBSS media

Group no 1

All teeth (15) PDL cells were submitted to histologic investigation after immersion in HBSS immediately post-extraction for 1h. Nearly 65.75% of cells were vital since it is not or mildly stained. On the other hand the remaining cells 34.24% were deeply stained indicating that they were non vital (Table 1).

Group no 2

Teeth of this group were immersed in HBSS immediately post-extraction for 4 hours. Vitality of periodontal ligaments cells were investigated and showed that 48.25% of cells were vital. The remaining cells (52.75%) were non vital (Table 1).

Group no 3

Teeth of this group were immersed in HBSS media for 6 hours post-extraction. The histological investigation for the detached periodontal ligament cells showed that nearly 38.08% of cells were vital and the remaining cells 62.92% were non vital (Table 1).

It was found that there is an inverse relation between storage time and PDL cell survival since the cells counts gradually decreased by the increase of storage time. Furthermore, there are highly significant differences between the numbers of PDL cells counted in the three groups (Table 1).

Point of comparison	1h storage time (Group 1)	4h storage time (Group 2)	6h storage time (Group 3)
N	15	15	15
Max. vital cells no. %	80.76	65.47	43.40
Mini. Vital cells no. %	51.61	34.62	30.00
Standard Error (SE)	2.02615	2.38061	1.01549
Standard Deviation (SD)	7.84726	9.22007	3.93296
Mean %	65.7507	48.2513	38.0840
Mean difference	1:2 = 17.49933*	2:3 = 10.16733*	1:3 = 27.66667*
Significance	1:2 = 0.000	2:3 = 0.000	1:3 = 0.000

Table 1: Effect of storage time on PDL cells vitality of teeth immersed in HBSS media

Histological investigation of PDL cells immersed in Egg white and milk mixture

Group no 4

Cells vitality of this group were histologically evaluated after immersion immediately post-extraction and storage for 1hour in Egg white-milk mixture. Digital counting showed that 49% of cells were vital while the remaining cells (51%) were dead (Table 2).

Group no 5

Histological investigation of this group teeth PDL cells were done after storing in Egg white-milk mixture immediately post-extraction for 4hours. However, it was found that 49.94% of cells were vital and the remaining cells (50.06%) were non vital (Table 2).

Group no 6

Table 3 showed that nearly 47.67% of cells of PDL of teeth immersed in Egg white and milk mixture immediately post-extraction and stored for 6 hourswere vital. On the other hand the remaining cells 52.33% were non vital.

As regard to storage time effect on PDL cells survival, teeth stored in Egg white-milk mixture histologic investigation showed low difference in favor of group 5 (50.06%) on the expense of group 4 (49%) and group 6 (47.67%). However, this increase in vital cells number from group 4 to group 5 was statistically insignificant while the decrease in cells number from group 5 to group 6 was significant (Table 2).

Point of comparison	1h storage time (Group 4)	4h storage time (Group 5)	6h storage time (Group 6)
N	15	15	15
Max. vital cells no. %	54.67	53.23	52.06
Mini. vital cells no. %	43.83	46.67	44.14
Standard Error (SE)	0.83105	0.50543	0.71738
Standard Deviation (SD)	3.21865	1.95751	2.77841
Mean %	49.0033	50.0640	47.6733
Mean difference	4:5 = -1.06067	4:6 = 1.33000	5:6 = 2.39067*
Significance	4:5 P = 0.289	4:6 P = 0.185	5:6 P = 0.02

Table 2: Effect of storage time on PDL cells vitality of teeth immersed in egg white-milk media

Comparing the effects of storage time on PDL cells vitality of teeth stored in HBSS vs egg white-milk media, it was found that after 1h there is a highly significant difference on favour of HBSS that showed 65.75% of cells were viable while egg white reported that 49% of PDL cells were vital. However, after 4h storage time this difference is gradually decreased and moreover converted in favour of egg white-milk mixture but without statistical significance. The irony is that this difference increases dramatically for the benefit of milk egg mixture (47.7%) compared to HBSS (38.1%) which significantly difference after 6h storage time (Table 3).

Comparison point	Group	N	Min. cells no. %	Max. cells. no. %	Stand. Err. (SE)	Stand. Devi. (SD)	Mean %	Mean diff.	Sig.
1h storage time.	Group 1 HBSS	15	51.61	80.76	± 2.02615	± 7.84726	65.7507	± 16.74733*	P = 0.000
	Group 4 E.M.MIX.	15	43.83	54.67	± 0.83105	± 3.21865	49.0033		
4h storage time	Group 2 HBSS	15	34.62	65.47	± 2.38061	± 9.22007	48.2513	± 1.81267	P = 0.373
	Group 5 E.M.MIX.	15	46.67	53.23	± 0.50543	± 1.95751	50.0640		
6h storage time	Group 3 HBSS	15	30.00	43.40	± 1.01549	± 3.93296	38.0840	± 9.58933*	P = 0.000
	Group 6 E.M.MIX.	15	44.14	52.06	± 0.71738	± 2.77841	47.6733		

Table 3: Comparison between the effect of HBSS media and egg white-milk (E.M.MIX) on PDL cells vitality at different storage times

Discussion

Traumatic dental injuries in children and adolescents are a common problem. Several studies have reported that the prevalence of these injuries has increased during the past few decades [32]. Tooth avulsion represents considerable percentage of all primary and permanent dentitions traumatic injuries [2-4,33]. It represents a potential threat for affected teeth periodontium and pulp so it requires quick emergency intervention. Since the increase in dry time can leads to lack of blood supply, nutritional supplements and humidity. In addition to bacterial contamination and consequently deterioration of the pulp and periodontal ligament cells [5,6,34].

Immediate replantation of the avulsed tooth is the treatment of choice to re-establish the natural nutrient supply of the periodontal ligament cells and enhance the healing process [35]. So maintenance of the avulsed tooth in a compatible storage medium to keep these cells survival before replantation in the socket is the key of success [9]. HBSS storage medium has the best approved osmolality, PH value and chemical composition [16]. It considered of choice for immediate storage of PDL of avulsed tooth since it preserve the vitality, mitogenicity and clonogenic capacity of PDL cells. Unfortunately, it is expensive, not available easily and sometimes became far away from the place of trauma [6,17]. The present study evaluated a novel, cheap, and commercially available mixture of Egg white-milk in maintaining the viability of (PDL) cells of intentionally extracted animal teeth. This technique mimic the clinical scenario of teeth avulsion. However, this mixture was compared to HBSS at different storage times using of Trypan Blue Exclusion Technique and digital counting of vital and non-vital cells.

In the present study, it was obvious that viability and survival of periodontal ligaments cells stored in HBSS decreased on increase of storage times. This finding was in agreement with Krasner., *et al.* who stated that HBSS is effective in preserving periodontal ligament cells of avulsed teeth, renew the degenerated periodontal ligament cells and maintain a superior success rate if an avulsed tooth is soaked in them for 30 minutes [25]. Furthermore, Krasner., et al. and Ashkenazi., et al. reported that, the pH balanced at 7.2 and osmolality of 320m Osm/kg allowing proper supply of different cell need to prevent starvation and dehydration up to replantation [35,36].

Teeth were stored immediately post extraction in HBSS solution for 4h and 6h, showed about gradual decrease in PDL valible cells numbers reaching to 48.25% and 38.08% respectively. However, the decreased number of viable cells may be referred to beginning of HBSS water loss especially storage of PDL cells was at room temperature not in incubation conditions as recommended [37,38]. This type of storage mimic the clinical scenario of tooth avulsion in trauma places. However, the decreased number of viable cells may be attributed also to HBSS nutrients starvation, although it still challenge for viability as there is no total cell death. These results came in harmony with the studies supported the fact that HBSS is prepared for immediate use at 37 °C in a controlled incubator,otherwise, it may came inferior to some other media [9,37-40].

Approximately Egg white-Milk mix has no previous studies concerning its effects on PDL cells viability. However, milk and egg white have scientific evidences of use as a solo storage medium. Moreover, they reported different results as they were tested under different situations. However, the viability of PDL cells of teeth stored in Egg white-milk mixture was competitive to viability of cells stored in HBSS in different conditions of storage times.

Viable PDL cells of teeth stored in Egg white-milk mixture for one hour immediately post extraction were 49% lower than viable cells count (65.75%) survived on storage in HBSS in the same conditions. This may be attributed to the fact that HBSS is classified as excellent media for immediate use [23]. However, for longer storage time (4-6h) milk may be superior to HBSS media. Furthermore, egg white alone was reported as good storage media showed better incidence of repair than milk for up to 6-10 h [1,20,23].

The result of the present study through light on the significance of adding white egg to milk to prolong the short term effect of milk. Blomlöf., *et al.* reported that milk is a compatible short-term storage medium for teeth if they were placed in it within 15 to 20 min of being avulsed [41]. Milk has a pH of 6.5 to 7.2 and osmolality of 270 mosmol kg-1, which is similar to extracellular fluid. Also McCulloch., *et al.* stated that Milk can potentially maintain PDL cell viability for up to 2 hours [42]. Moreover, Khademi., *et al.* stated that no significant difference between egg white and HBSS at storage time of one hour has been established but egg white was more suitable than water and milk in this study [31].

PDL cells stored in Egg white-Milk mixture for 4& 6 hours immediately post-extraction reported higher percentages of viable cells counts (50.06% & 47.67% respectively) than that of HBSS. These results can be interpreted in the light of the synergistic effect of egg white-milk mixture rich in many essential contents. Since milk has suitable physiological pH & osmolality, in addition to water; it is rich in many growth factors and nutrientsas proteins, vitamins and minerals. These contents are needed by cells to prevent starvation, dehydration in addition to proper pH and osmolality. As well as being free of bacteria that decrease infection possibility [32,43]. Bloml., et al. stated that milk is an excellent storing solution for 6 hours when immediate replantation is not possible [22]. Also egg white has low bacterial contents and rich in the essential contents especially high protein contents and essential amino acids that needed to promote PDL cells healing and mitosis [29]. Lindskog., *et al.* reported that milk loses its effect if used as a solo storage medium after two hours [44]. In the same context, Khademi., et al. reported that teeth stored in egg white for 6 to 10 h had a better incidence of repair and lower surface resorption than those stored in milk for the same time [20].

Conclusion

Based on this study finding; it could be concluded that:

1. Egg white-Milk novel mixture showed a competitive properties to HBSS as a storage media for immediate use.

2. Egg white-Milk mixture has significant superior properties and has the ability of replenishment of metabolites in depleted cells more than the approved evidences of HBSS 38,39 after 4 and 6h storage time.

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