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Assessment of Periodontal Ligament Fibroblast Viability in Dymatize® Elite Casein as a Transport Medium for Avulsed Teeth at Different Time Intervals (An *in - vitro* study)

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Abstract

Aim of the study: To evaluate the effectiveness of a sports dietary supplement, which is Dymatize® Elite Casein (micellar casein), as a storage medium for avulsed teeth compared to Hank's balanced salt solution at three-time intervals.

Material and method: One hundred teeth freshly extracted for orthodontic treatment. Were divided into Hank's balanced salt solution, Dymatize® Elite Casein, a Dry group, and a control that tested directly after extraction. Experimental groups' teeth were immediately immersed in the media and subdivided into three groups at time intervals (1 hr, 4 hrs, and 8 hrs). Cells of PDL were examined after being scraped, and labeled with trypan blue using a light microscope and a hemocytometer. The data were statistically analyzed, using one-way ANOVA with Graph Pad Prism 7. And, Post Hoc Tukey's test performed inferential to compare each test group to all other groups.

Results: All groups showed highly significant changes, except for Dymatize® and control at one hour, which showed non-significant differences. All experimental groups had highly significant differences after 4 and 8 hours.

Conclusion: The Dymatize® Elite Casein could serve effectively as a transport medium and preserve PDL viability over time ensuring replantation success. It is a good substitute with its superior properties.

Keywords: Dymatize® Elite Casein**,** Fibroblast viability, Storage media, Avulsed teeth.

Introduction

Facial trauma in children has significant psychological, esthetic, and functional effects on children. The decline in the incidence of dental caries has made traumatic dental injuries the greatest threat to dental health (Andreasen, 1981; Marcenes et al, 2001). A study by Attyia (2019) concluded that the majority of pediatric maxillofacial trauma was in the school-age group, and this can be explained by the fact that school-aged children engage in more activities in addition to the anatomical changes associated with facial growth that make them more susceptible to facial fractures, also verified that male children are roughly twice as affected as female children, which is explained by the fact that male children engage in more physical activity.

One of the complications of traumatic injuries to developing permanent teeth is devitalization of the pulp, which results in a concurrent cessation of the growth of the implicated tooth's immature roots (Al-Dahan et al, 2014). The maxillary central incisors are the most frequently affected teeth, due to their position in front and the protrusion that the eruptive process causes (Khalaf et al, 2021).

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Tooth avulsion can be defined as the displacement of the tooth from its socket that leads to damage to the periodontal ligament (PDL) structure and the tissues begin to dehydrate (Balto et al., 2015). The tooth must be replaced immediately within 5 min. but sometimes it is not possible, so the tooth is placed in a humid solution to prevent a dry environment, which leads to the need for a storage medium that closely replicates the oral environment to protect the periodontal ligament cells and preserve its vitality, which is a critical factor in replantation success (Gomes et al., 2009). The existence of cells able to multiply on the injured part of the root will determine how successful tooth replantation will be (Staniowski et al, 2021). and The pulp of both primary permanent teeth, the periodontal ligament, and other nearby healthy tissues can all be used to obtain dental stem cells, Permanent teeth extracted for orthodontic treatment, are all readily available sources of dental stem cells (Al-Sarraj & Saliem, 2014), and ensures the ability for replantation success. PDL is a potential source of stem cells, and by tissue engineering, cells with bioactive agents (e.g. growth factors) used to regenerate damaged and diseased tissues, periodontal ligament stem cells play a significant role in the regeneration of periodontal tissues (Safi et al, 2020).

Moreover, the health of the periodontal ligament cells is essential to the success of reimplantation, therefore tooth structure should be kept for as long as possible in a sufficiently moist environment (Khan & Sharma, 2020). In addition, the knowledge of clinicians of the stability changes during healing phase able them to choose the optimum time for functional loading (Alsheikhly and Bede, 2019).

The storage solutions should be capable of saving the PDL vitality until replantation can be done, the pH and osmolality properties of this solution are important. In addition, the way that the tooth is transported (Poi et al, 2013), bacterial invasion into underlying connective tissues has been linked to loss of epithelial integrity, which could intensify the inflammatory response and cause tissue damage (Abdulkareem et al, 2018). The properties of an ideal storage media should concentrate on keeping the remaining periodontal ligament healthy and strong (Adnan & Khan, 2015), it's available for use at the site of the accident and not need time for preparation, be inexpensive, have the essential nutrients, low bacterial contents, sterile, antimicrobial, no antigen-antibody reaction, neutral pH, physiological osmolality and keeps the cells vitality (Jain et al., 2015).

Multiple storage media have been suggested, starting with placing the avulsed tooth in a patient's mouth by Axhusen, (1948), using milk as a storage medium (Mahendra et al, 2022). Albumin (egg yolk) has been suggested by Layug et al, (1998) (Adnan & Khan, 2015). Later on, Martin & Pileggi (2004) used propolis for avulsed teeth as a transport medium. Further, more studies focus on types of storage media that are used as emergency management for avulsed teeth.

The majority of previous storage media were used by a person who was likely at the site of the accident, teachers, caretakers, or parents. Some of these media require preparation near the site of the

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accident, and we know the factor of time is important. The originality of this research is the use of a sports dietary supplement which is Dymatize® Elite Casein (micellar casein) as a medium to save avulsed teeth and enhance the treatment outcomes. The current study aimed to assess the dry time effect on the health of periodontal ligament cells and to evaluate the effectiveness of Dymatize® Elite Casein on periodontium cell vitality at different time intervals in comparison to Hank's balanced salt solution.

Materials and Methods

Teeth have been extracted and collected freshly from patients within orthodontic treatment (ethical approval number is 836323), taking care of the root surface by holding only from the crown portion by tweezer or forceps (Mahendra et al, 2022). This study was done with one hundred teeth samples divided into three main experimental groups (30 samples each) (Jacob Cohen, 1988). Dry, Gg, and HBSS, in addition to a control group, were tested directly after extraction and treated with collagenase and neutral protease enzymes in phosphate-buffered saline (PBS) (Shingare & Chaugule, 2020). Each group was subdivided into three-time intervals (1 hr., 4 hr., and 8 hr.) (Shingare & Chaugule, 2020; Sanghavi et al, 2013).

All the teeth were placed in their appropriate storage medium right after extraction in experimental groups to maintain the same basic standards (Babaji et al, 2017). In this study, the experimental groups were (30 teeth) for each:

- **HBSS group:** In this group, teeth were immersed in Hank`s balanced salt solution directly after extraction and stored for different time intervals (1 hr., 4 hr., and 8 hr.), 10 teeth for each time interval (Patel et al, 2022).
- **Dymatize® Elite Casein (micellar casein) group:** In this group, teeth were immersed in this solution as a storage medium for time intervals of 1hr.,4hr., and 8 hr., 10 teeth for each time interval.
- **Dry group:** In this group, teeth were bench-dried for (1 hr.,4 hr., and 8 hr.) without the use of storage media. For each time interval subgroup, 10 teeth were used (Adnan & Khan, 2015; Shingare & Chaugule, 2020).

Each tooth in all groups was treated separately in a 15 ml. falcon tube with 2.5 ml. solution (0.1 ml. of 0.2 mg/ml of collagenase, then 0.1 ml. of 2.4 mg/ml of neutral protease was added, to the tube) in 2.3 ml. phosphate buffer saline and 30 minutes of incubation at 37°C (Shingare & Chaugule, 2020). Fetal bovine serum (50 ml) was added to each tube following incubation (Babaji et al, 2017). Apical two-thirds of the root surfaces were scraped with Bard-Parker blade No. 15 (Surgical) in a petri dish to obtain the PDL cells (Mahendra et al, 2022), then all tubes were centrifuged for five minutes at a speed of 1000 rpm, and the supernatant was collected using sterile micropipettes. Five to fifteen minutes were given for the test tube to leave stand (Babaji et al, 2017). Using a Micropipette of 5–50 µl, add a tiny volume of the Trypan blue–cell suspension. The slide chamber was prepared with 25

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ml. of the stained solution. Using a micropipette, the stained solution was added to both chambers of Neubauer's chamber, covered with a coverslip, and allowed to settle for 1-2 minutes. It should not be left longer as the cells may die and so take up the dye (Mahendra *et al.*, 2022).

Determination of PDLcell numbers:

The PDL cells were then examined using an optical microscope (Mahendra et al, 2022). And after being labeled with 0.4% trypan blue (Shingare & Chaugule, 2020), their vitality were tested at a 40X magnification using a light microscope and a hemocytometer (Sanghavi et al, 2013). The slide chamber is made up of two squares on each side with four squares on each. Viable and non-viable cells were counted separately by Trypan blue staining. Viable cells shine, while dead cells seem dark in the light microscope. The cell number for each unit volume was calculated as: (Khinda et al, 2017).

Viable Cells per ml = The counted viable cell per square ×dilution factor ×10^4

The viable cell percentage was calculated as: (Mahendra et al, 2022).

Cell Viability (%) = (Total no. of cells - No. of non-viable cells/ Total no. of cells) ×100

The viable cells are shiny which the cells have an intact membrane and the non-viable cells stain blue because without an intact membrane, take up the coloring agent. Then the difference between each storage media and their effectiveness should be assessed (Zhang et al, 2021).

Statistical Analysis

Data was statistically analyzed with GraphPad Prism 7 (Dotmatics, Boston, USA) (Al-Shammari et al, 2020). One-way ANOVA was used to examine the number of viable cells from each storage media, and the Post Hoc Tukey test was performed to compare each test group to all other groups.

RESULTS

Control group: The fibroblast cell's viability was tested directly, and the mean percentage was 87.4 %, an image taken under the microscope after trypan blue staining, shown in Figure 1.

Experimental groups: The data from the experimental study was analyzed using Tukey's multiple comparisons test with a level of significance at $p < 0.05$ to reveal the impact of using different storage media for the preservation of vitality of PDL cells for saving avulsed teeth and to determine the dry time effect on these cells. The control group (Direct), in which the cell viability has been evaluated directly after extraction (0 min.) as normality to compare with teeth stored in different types of storage media in the experimental groups (Table1).

Viability of PDL cells for all groups in each time interval

After 1 hour storage in each media: The mean percentage of PDL cell viability for all experimental groups and comparison between them after 1 hr. storage using F-test analysis of variance (ANOVA), which was highly significant ($p \leq 0.01$), as demonstrated in Table 1.

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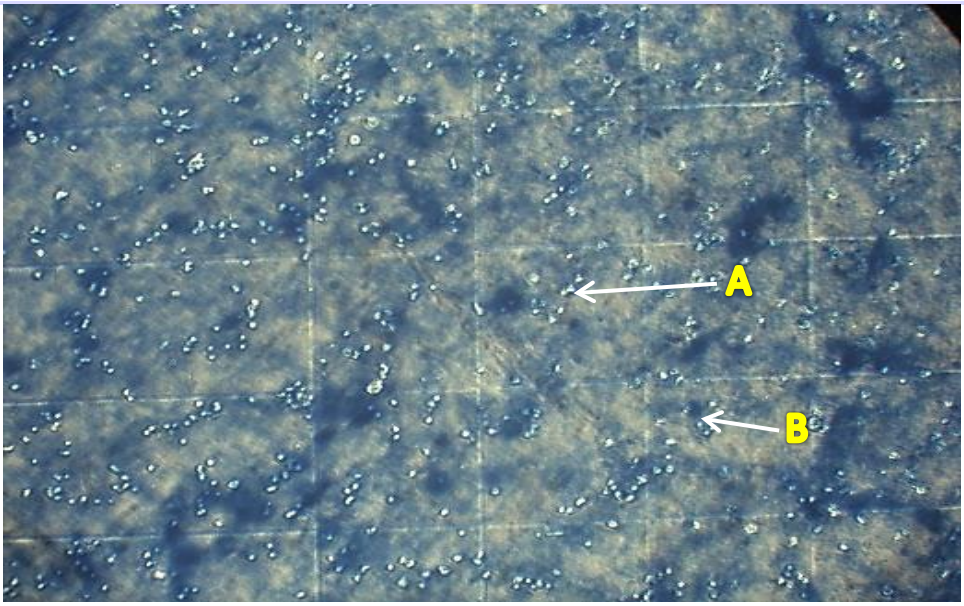

Figure 1: Viable cells of PDL in the control group (A= viable cells shiny; B = nonviable cells dark)

The mean difference between any two groups within 1 hr. time interval of different storage media and the control showed highly significant differences ($p \leq 0.01$), on the other hand, the differences between DM vs. Direct exhibited non-significant values ($p \geq 0.01$) by using Post Hoc Tukey's test (Table 2).

Table 1: Mean % for PDL cell viability and comparison between all groups at three-time intervals (1hr., 4 hr., and 8 hr.)

Group	N	Mean % at 1hr.	SD % at 1hr	Mean % at 4hr.	SD % at 4hr.	Mean % at 8hr.	SD % at 8hr.
HBSS	10	57.26	9.818	26.12	3.372	13.94	4.258
DM	10	80.67	6.131	57.83	11.21	36.55	12.85
Dry	10	53.18	13.31	20.66	2.876	2.91	0.2726
Direct	10	87.4	5.066	87.4	5.066	87.4	5.066
F-test (ANOVA)		21.64		209.1		249.6	
p-Value		0.0001 (HS)		0.0001 (HS)		0.0001 (HS)	

N: Number of samples
HBSS: Hank's balanced salt solution
DM: Dymatize® Elite Casein
Dry: Without storage medium
Direct: Immediately after extraction
HS: Highly significant at $p < 0.01$

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Table 2: Post Hoc Tukey's test for fibroblast viability comparison between any two groups after 1 hr. storage

Post Hoc Tukey's test	Mean Diff.	p-*value*	Significant
HBSS vs. DM	-23.41	0.0001	HS
HBSS vs. Dry	4.08	0.8962	NS
HBSS vs. Direct	-30.14	0.0001	HS
DM vs. Dry	27.49	0.0001	HS
DM vs. Direct	-6.73	0.5799	NS
Dry vs. Direct	-34.22	0.0001	HS

S: Significant at $p < 0.05$ NS: Not significant at $p > 0.05$ HS: Highly significant at $p < 0.01$

HBSS: Hank's balanced salt solution
Dry: Without storage medium

DM: Dymatize® Elite Casein
Direct: Immediately after extraction

After 4 hours storage in each media: The mean percentage of PDL cell viability for all experimental groups after 4 hr. using F-test analysis of variance (ANOVA) and comparisons between them were highly significant differences $p < 0.01$.

While using Post Hoc Tukey's test, all groups showed highly significant differences ($p \leq 0.01$), and a non-significant difference ($p > 0.05$) between the HBSS and Dry group (Table 3).

After 8 hours storage in each media: The mean percentage of PDL cell viability after 8 hours for the experimental groups in comparison with each other and the control using F-test analysis (ANOVA) showed highly significant differences ($p \leq 0.01$) (Table 1).

The mean differences between experimental groups within 8 hr. time intervals showed highly significant differences ($p \leq 0.01$) between all groups as demonstrated in (Table 4).

Different media after 8 hours' storage, the mean viable cell percentage between all groups with the control after 8 hours (Figure 2).

Table 3: Post Hoc Tukey's test for fibroblast viability comparison for different storage media after 4 hr. storage

Post Hoc Tukey's test	Mean Diff.	p-*value*	Significant
HBSS vs. DM	-31.71	0.0001	HS
HBSS vs. Dry	5.46	0.2690	NS
HBSS vs. Direct	-61.28	0.0001	HS
DM vs. Dry	37.17	0.0001	HS

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DM vs. Direct	-29.57	0.0001	HS
Dry vs. Direct	-66.74	0.0001	HS

S: Significant at $p < 0.05$ HS: Highly significant at $p < 0.01$ NS: Not significant at $p > 0.05$

HBSS: Hank's balanced salt solution DM: Dymatize® Elite Casein

Dry:Without storag medium Direct: Immediately after **extraction**

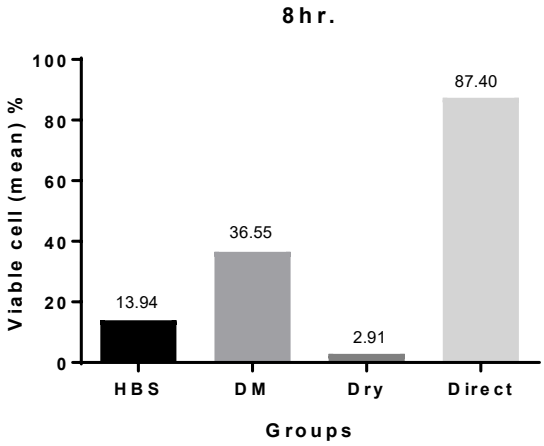
Table 4: Post Hoc Tukey's test for fibroblast viability comparison for different storage media between any two groups after 8 hr. storage

Post Hoc Tukey's test	Mean Diff.	*p*- value	Significant
HBSS vs. DM	-22.61	0.0001	HS
HBSS vs. Dry	11.03	0.0040	HS
HBSS vs. Direct	-73.46	0.0001	HS
DM vs. Dry	33.64	0.0001	HS
DM vs. Direct	-50.85	0.0001	HS
Dry vs. Direct	-84.49	0.0001	HS

HS: Highly significant at $p < 0.01$ NS: Not significant at $p > 0.05$

HBSS: Hank's balanced salt solution DM: Dymatize® Elite Casein

Dry: Without storage medium Direct: Immediately after extraction



HBSS: Hank's balanced salt solution Dry: Without storage medium

DM: Dymatize® Elite Casein Direct: Immediately after extraction

Figure 2: The mean value (%) of PDL cell viability after (8hr.) for all groups

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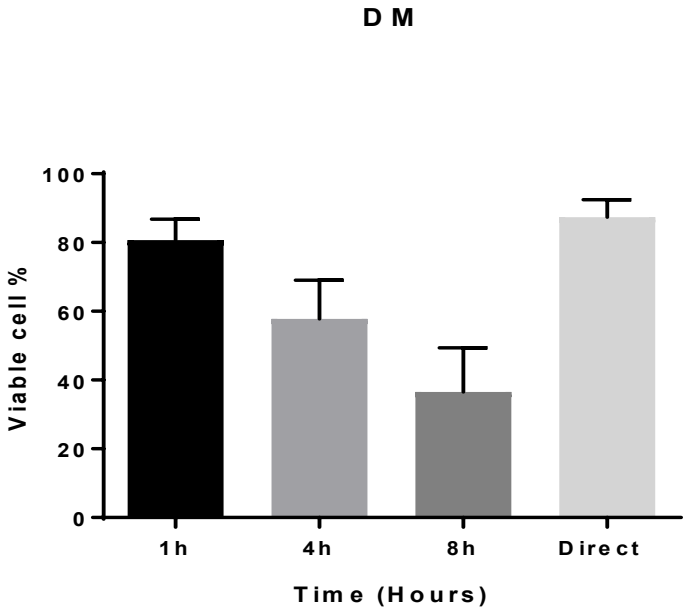
The same storage media at different time intervals

The mean viable cell % of the PDL for the HBSS group, the Dymatize® group, and the dry group of the same medium at three-time intervals 1 hr., 4 hrs., and 8 hrs. was evaluated using F- test analysis of variance (ANOVA), it showed a highly significant difference $p \leq 0.01$ for all the experimental groups except the Direct vs. Dymatize® at 1 hr. time interval which showed non-significant differences $p \geq 0.05$ (Table 5) (Figure 3).

Table 5: Mean value % for PDL cell viability of DM group at different storage time intervals

Group	N	Mean (%)	SD (%)	Minimum Score(%)	Maximum Score(%)	F-test (ANOVA)	P-Value
Direct	10	87.4	5.006	84	99.9	60.53	0.0001 (HS)
DM 1hr.	10	80.67	6.131	74.4	95.2		
DM 4 hr.	10	57.83	11.21	36.1	66.5		
DM 8 hr.	10	36.55	12.85	21.3	58.2		

HS: Highly significant at $p < 0.01$ DM: Dymatize® Elite Casein Direct: Immediately after extraction



DM: Dymatize® Elite Casein Direct: Immediately after extraction

Figure 3: The viable cells % of DM at different time intervals

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Discussion

Dymatize® Elite Casein (micellar casein) is an original medium that was used in the current study for the first time. It is a very popular supplement for athletes, casein is one of two proteins in milk, high in calcium and low in cholesterol, it's one of the protein products with amino acids like leucine. Amounts per serving are; 25 g. protein, 2 g. carbs, 1.5 g. fat, 5.3 g. Branched Chain Amino Acids, and 2.3 g. leucine, this high protein content may preserve cell viability by supplying important ingredients that are essential for cell nourishment plus other contents. In addition, it can be readily prepared for use as manufacturer instructions and can be available at times when needed on accident sites such as schools or playgrounds.

After 1- hour storage time the results for the HBSS medium when compared with the control showed a non-significant difference. There was no- significant difference between DM and the control group which revealed that the DM medium may be more efficient in preserving cell viability than HBSS. The HBSS consists of calcium chloride, magnesium sulfate anhydrous, potassium chloride, sodium bicarbonate, monobasic potassium phosphate, sodium chloride, and D-glucose. It has an osmolality of 280 milliosmol per kilogram and a pH of 7.4 **(** Navin et al, 2015) .

Although HBSS has been designated as the gold standard of preservation media for avulsed teeth (Fagundes et al, 2018), there were still limitations. Thus there is a need to study new materials that are easily available and prepared, more practical, and can preserve PDL fibroblast viability for longer periods of storage to ensure successful replantation. It should concentrate on the duration between the tooth avulsion from the alveolar socket and its insertion in any transport medium which is crucial to the success of replantation (Mahendra et al, 2022).

The extraoral dry time, that's to say the time extended from tooth avulsion till replantation or placement in a transport media. It is recommended to insert the tooth gently into the socket, and the optimal time during which PDL cells maintain high viability is thought to be 15 to 20 minutes (Fagundes et al, 2018). If time is exceeded, undifferentiated mesenchymal cells lose the capacity to transform into the fibroblasts required for repair (Ashkenazi et al, 2000). This agreed with our results that showed a highly significant difference between the Dry group and the control, which ensured cell viability suppression after a 1 hr. dry period when kept without storage media. These results may be an indication that the DM medium was able to maintain the viability of PDL fibroblast cells more than other media and better than the HBSS medium after 1 hour.

The DM medium has superiority over other media in maintaining cell viability after 4 hours, although there was a high significant difference with the control which may be due to high protein contents that are essential for cell nourishment. Secondly comes the HBSS group. However, HBSS was still able to save fibroblast viability and this agreed with Ashkenazi et al (2000) who determined that HBSS was a suitable storage medium after 4 hours of dry time as long as live cells were present (Ashkenazi et al, 2000). The mean

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percentage of viable cells that were kept dry in the experimental group remained the lowest among all groups in preserving the viability of fibroblast cells of the extracted teeth. This had been supported by Zohreh Ahangari et al, (2013) who showed no vital PDL cells remained after two hours dry (Ahangari et al, 2013).

After 8 - hours storage time storage of teeth in all media showed a comparable decrease in the mean percentage of viable cells with the control which showed a high significant difference, this was compatible with Malhotra's (2011) findings that showed most storage media gradually lost their therapeutic efficacy over time.

The results showed that the mean percentage of viable cells for the DM group was the highest value among other groups, which may be explained by the nutritional contents of this medium and high protein as stated above.

Although HBSS medium preserves 70% fibroblast vitality after 96 hours as was concluded by Hiltz & Trope (1991), in this study after 8 hours there was a major decrease in cell viability as results showed a highly significant difference with the control group. Thus, DM medium could also aid in preserving fibroblast cells and prevent lysis when immediately immersed in it till replantation. The mean viable cell percentages were more than that of the HBSS group.

Results showed that a high decline in the mean percentage of fibroblast viability after 8 hours left dry without storage media which leads to cell damage and loss of their ability to repair and disturbs the tooth replantation process. According to Navit (2017), dry storage media was the least preferred form for avulsed teeth. In general, media speeds up the healing process by re-establishing the normal supply of nourishment to the periodontal ligament cells on the root surface, reducing additional damage (Mahendra et al, 2022).

Cell viability within the same storage media at various time intervals

The current study showed highly significant differences in the DM group between different storage time intervals, the mean percentage value of PDL cells after 1 hr. was 80.67 %. When compared with the control group in which the fibroblast cells of extracted teeth immediately evaluated for viability showed 87.4 % this value was close to that of the DM group after 1 hr. storage.

The mean percentage values after storage for 4 hrs. and 8 hrs. were 57.83 % and 36.55 %, respectively. This made it a good transporting medium, moreover, it was available commercially as a popular supplement that could be readily prepared as manufacturer instructions.

In the current study, the cell viability examination in the Dry group for the same time periods as experimental storage media for comparison reasons, while previous studies mainly take a single time period as a bench dry group (Mahendra et al, 2022; Shingare & Chaugule, 2020). Results at three-time intervals; 1hr., 4 hr., and 8 hr. showed highly significant differences in the same group (Dry group). The mean percentage values for PDL cell viability were 53.18 %, 20.66 %, and 2.91 % in a sequence of time periods.

Obviously, it has been noticed that cell desiccation occurred and subsequent loss

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of viability of PDL cells with time and cell damage had taken place. This was in agreement with Khinda et al, (2017) who found that more than 30 minutes' dry had an efficient impact on the differentiation ability of mesenchymal cells into fibroblasts necessary for repair.

However, when recommending a suitable transfer media for an avulsed tooth, it is crucial to take the accident conditions and location into account, especially in an emergency (Venugopal, 2022). In general, media speeds up the healing process by re-establishing the normal supply of nourishment to the periodontal ligament cells on the root surface, reducing additional damage (Mahendra et al, 2022).

Limitation

1. The tooth samples should be collected and isolated from each other and directly immersed in the specific medium, then the time period elapsed differed in the experiment from one sample to another.
2. After the specific time of storage, cell viability should be evaluated directly, otherwise it will die before reading the slide.

Conclusion

This study was concluded that the Dymatize® Elite Casein had properties as a transport medium. In addition, its availability, usability, and convenience of use, as an athlete's supplement eases, and is readily prepared.

After 1 hr. storage, the DM medium had a better effect than HBSS in preserving the viability of PDL cells. Also a more efficient effect after 4 hrs. storage, in comparison to HBSS medium which were less efficient although both had a close effect. DM is capable of preserving viability for up to 8 hours indicating that the DM medium had the highest value among the other groups.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Data Availability Statement

Data are available from the authors upon reasonable request.

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