

# Clonogenic Capacity of Periodontal Ligament Cells Stored in Different Storage Media

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## ABSTRACT

**Objectives:** We examined the effects of storage in Hank's balanced salt solution (HBSS), low-fat milk, soy milk, and aloe vera extract on the clonogenic capacity of human periodontal ligament (PDL) cells. The Dulbecco's modified Eagle's medium (DMEM) was used as control.

**Materials and methods:** Human teeth that are extracted for the orthodontic purpose were collected and the periodontal ligament tissue was cultured. Cultured experimental PDL cells were exposed to different experimental solutions for 24 hours. To evaluate the clonogenic capacity of the stored cells, cells were inoculated into 96-well plates at a concentration of one viable cell/well. For each tested group, 96 replicates were plated and grown for 3 weeks in a culture medium at 37°C in humidified air containing 7% CO<sub>2</sub>. Under these conditions, discrete colonies arising from a single cell that covered 70–100% of the well were counted under light microscopy (×200). The percentage of cells with clonogenic capacity was calculated as the number of colonies formed/number of cells seeded × 100. Thus, the clonogenic capacity reflected the likelihood that each colony arose from a single cell. The experiments for 2 and 8 hours were repeated three times (three 96-well plates), and for 24 hours, five times (five 96-well plates).

**Results:** The highest clonogenic capacities were found in cells stored in HBSS (15.3 ± 1.15) and aloe vera extract (12.3 ± 2.5) followed by low-fat milk (10.3 ± 1.5). Soy milk showed least clonogenic capacity (3.6 ± 1.5). The Dulbecco's modified Eagle's medium, HBSS, and aloe vera showed similar statistical significance ( $p = 0.092$ ).

**Conclusion:** Highest clonogenic capacity was found in cells stored in HBSS, aloe vera extract, and low-fat milk. Soy milk showed least clonogenic capacity.

**Keywords:** Aloe vera extract, Clonogenic capacity, Hank's balanced salt solution, Periodontal ligament cells, Soy milk.

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## INTRODUCTION

The World Health Organization has defined avulsion as the complete displacement of a tooth from its alveolar socket due to traumatic injury.<sup>1</sup> Tooth avulsion is one of the most severe dental traumas and its prevalence is approximately 1–16% of all traumatic injuries to the permanent dentition.<sup>2</sup> Andreasen and Daugaard-Jensen predicted that the incidence of these injuries might surpass the incidence of dental caries.<sup>3</sup> The etiological factors are trauma after fighting, sports, falls, and bumps against hard objects or the floor.<sup>4</sup>

Avulsion causes rupture of the neurovascular bundle, which leads to loss of pulp vitality<sup>5</sup> and necrosis of the periodontal ligament. Necrosed periodontal ligament (PDL) cells in the replanted tooth promote the inflammatory process and in severe situations lead to replacement root resorption and loss of tooth.<sup>6</sup>

Immediate replantation is the ideal treatment for maintaining the viability of PDL cells. However, immediate replantation is not possible because of various associated factors such as the person's conscious state, lack of first aid knowledge, informed consent issues, and lack of confidence in strangers gathered at the scene of accident. Success of replantation was initially thought to be associated with the speed with which the tooth is replanted but now researchers have demonstrated that the storage medium is one of the most important factors than the extra oral time.

A storage medium may be defined as a physiological solution that closely replicates the oral environment to help preserve the viability of PDL cells following avulsion.<sup>7</sup> A storage medium should provide physiological pH and osmolarity, should have antibiotic and anti-inflammatory properties and clonogenic and mitogenic properties, should be easily available at the site of accident, and

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should be economical.<sup>8</sup> Unfortunately, such an ideal storage medium has not been discovered yet.

Studies have demonstrated that when extracted teeth were dried, there was a rapid decrease in viable cells within 15 minutes, and by 2 hours no viable cells were present in the PDL.<sup>9</sup>

Regeneration of the PDL after replantation is related to preservation of the viability of the mixed population of PDL cells that attached to avulsed teeth. In the past, emphasis was placed on assays of cell membrane integrity (vitality) and cell attachment (plating efficiency). However, the ability of PDL cells to proliferate and form clones (clonogenic capacity) indicates the presence of viable progenitor cells and may be related to clinical survival than assays of PDL cell vitality or cell attachment.<sup>10</sup>

The purpose of this study was to investigate the effects of storage media, Hank's balanced salt solution (HBSS), low-fat milk, soy milk, and aloe vera extract, on the clonogenic capacity of human PDL cells at 2 hours, 8 hours, and 24 hours.

## MATERIALS AND METHODS

The study was conducted in the Department of Pedodontics and Preventive Dentistry, College of Dental Sciences, Davangere, in association with the Department of Oral and Maxillofacial Pathology and Microbiology, Maratha Mandal's Nathajirao G. Halgekar Institute of Dental Sciences and Research Center, Belagavi. This study was approved by the institutional review board, College of Dental sciences, Davangere.

### Cell Culture of Human PDL Cells

Periodontal ligament cells were obtained from clinically healthy premolars extracted for orthodontic purposes. Extraction was performed as atraumatically as possible, and a tooth was washed in sterile saline solution to wash out residual blood. The tooth was stored in a conical tube filled with HBSS. The tooth was held with forceps at the coronal region, and the PDL cells were obtained by scrapping with a no. 11 scalpel blade from the middle thirds of the root surfaces. The tissues were split into small pieces and cultured in the Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. The PDL cells outgrown from PDL tissues expressed a fibroblast-like phenotype and were allowed to reach confluence and passed at a 1:2 ratio until they were used for the experiment.

### Experimental Groups

Experimental PDL cells were washed by phosphate-buffered saline, and these cells were exposed to different experimental solutions. The storage solutions used in the experiments were as follows: (1) group I: HBSS, (2) group II: low-fat milk, (3) group III: soy milk, and (4) group IV: aloe vera extract.

### Clonogenic Capacity

Cultured experimental PDL cells were exposed to different experimental solutions for 24 hours and PDL cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTT) assay. To evaluate the clonogenic capacity of the stored cells, cells were inoculated into 96-well plates at a concentration of one viable cell/well. For each tested group, 96 replicates were plated and grown for 3 weeks in a culture medium at 37°C in humidified air containing 7% CO<sub>2</sub> supplemented with growth factors (platelet-derived growth factors). Under these conditions, discrete colonies arising from a single cell that covered 70–100% of the well were counted under light microscopy (×200). The percentage of cells with clonogenic capacity was calculated as the number of colonies formed/number of cells seeded × 100. Thus, the clonogenic capacity reflected the likelihood that each colony arose from a single cell. The experiments for 2 and 8 hours were repeated three times (three 96-well plates), and for 24 hours, five times (five 96-well plates).<sup>11</sup>

### Statistical Analysis

Two-way ANOVA with repeated measurements and Tukey HSD were used for comparison of clonogenic capacity of four different storage media.

## RESULTS

Clonogenic capacity of HBSS, aloe vera extract, and low-fat milk (mean value 15.3 ± 1.15, 12.3 ± 2.5, 10.3 ± 1.5, 3.6 ± 1.5) was higher at 24 hours except soy milk that showed decrease in cell count

(Table 1). The Tukey HSD test (Table 2) showed that soya milk (*p* value 1.00), low-fat milk and aloe vera (*p* value 0.068), and aloe vera, HBSS, and control (*p* value 0.092) form statistically similar groups when it comes to capacity of PDL cells.

## DISCUSSION

This study was conducted to evaluate the clonogenic capacity of PDL cells stored in four different storage media supplemented with growth factors (periodontal ligament growth factor, PDGF). The clonogenic capacity of periodontal ligament fibroblasts (PDLF) estimates the proportion of progenitor cells in a population with extensive proliferative and colony-forming capacities. This may suggest the effectiveness of each medium to preserve the progenitor cells.<sup>12</sup>

The exact nature of the PDL cells responsible for repopulating the damaged root surface has not yet been clarified. However, fibroblasts are the most dominant, as well as the most easily cultured, cells in the PDL. These cells were therefore used in this study. PDLF from teeth of young adults were used to imitate the cell lining of avulsed teeth as closely as possible. The effectiveness

**Table 1:** Descriptive statistics

|                | Media        | Mean    | Std. deviation | n  |
|----------------|--------------|---------|----------------|----|
| After 2 hours  | Control      | 12.3333 | 0.57735        | 3  |
|                | HBSS         | 10.6667 | 0.57735        | 3  |
|                | Aloe vera    | 10.0000 | 2.00000        | 3  |
|                | Soya milk    | 9.3333  | 0.57735        | 3  |
|                | Low-fat milk | 8.0000  | 2.64575        | 3  |
|                | Total        | 10.0667 | 1.98086        | 15 |
| After 8 hours  | Control      | 14.0000 | 1.73205        | 3  |
|                | HBSS         | 14.0000 | 1.73205        | 3  |
|                | Aloe vera    | 12.6667 | 2.51661        | 3  |
|                | Soya milk    | 5.3333  | 4.16333        | 3  |
|                | Low-fat milk | 10.0000 | 2.00000        | 3  |
|                | Total        | 11.2000 | 4.03909        | 15 |
| After 24 hours | Control      | 15.3333 | 0.57735        | 3  |
|                | HBSS         | 15.3333 | 1.15470        | 3  |
|                | Aloe vera    | 12.3333 | 2.51661        | 3  |
|                | Soya milk    | 3.6667  | 1.52753        | 3  |
|                | Low-fat milk | 10.3333 | 1.52753        | 3  |
|                | Total        | 11.4000 | 4.65679        | 15 |

**Table 2:** Homogeneous subsets

| Measure_1    |   | Subset |         |         |
|--------------|---|--------|---------|---------|
| Media        | n | 1      | 2       | 3       |
| Soya milk    | 3 | 5.2500 |         |         |
| Low-fat milk | 3 |        | 9.2500  |         |
| Aloe vera    | 3 |        | 11.8333 | 11.8333 |
| HBSS         | 3 |        |         | 13.8333 |
| Control      | 3 |        |         | 14.2500 |
| Sig.         |   | 1.000  | 0.068   | 0.092   |

\*Mean values as per test

of storage media was evaluated after 2, 8, and 24 hours as these intervals were thought to be clinically relevant.

Hank's balanced salt solution is a popular storage medium that has been shown to be effective in preserving avulsed teeth for extended periods.<sup>13-15</sup> In this study, HBSS was found to be most effective storage medium in preserving the clonogenic capacity of cells after storage for up to 24 hours at 4°C. Ashkenazi et al.<sup>16</sup> studied mitogenicity and clonogenic capacities of the PDL fibroblast stored in ViaSpan, HBSS, a minimal essential medium (MEM), and a MEM supplemented with FCS and antibiotic (MEM-S) at 2, 8, and 24 hours and found that HBSS and a MEM-S supplemented with the growth factor (GF) were the most effective media for preserving the viability, mitogenicity, and clonogenic capacity of PDLF stored for 24 hours, which supports our study. In contrast to our results, study conducted by Lekic et al.<sup>17</sup> showed decrease in the clonogenic capacity of the storage medium with increase in the time. This may be attributed to difference in the method of study where they have studied clonogenic capacity of PDL cells without adding GF.

Aloe vera was the second best choice according to this study. Statistical significance was similar to HBSS and DMEM. Tudose et al.<sup>18</sup> studied regenerative properties of aloe vera juice on human keratocyte cell culture and found that the 50% aloe vera extract showed better cell growth at 48 hours, the reason for which could be its physiologic osmolarity; the osmolarity of aloe vera was found to be in a range of 280–300 mOsm/L and normal cell growth occurs at a osmolarity range of 230–400 mOsm/L. It contains 99% water and over 75 nutrients, which include 20 minerals, 19 amino acids, and 12 vitamins. The human body requires 22 amino acids to maintain good health, 8 of which are essential, as the body cannot synthesize them. All of these eight essential amino acids and 11 of 14 secondary amino acids are found in aloe vera. It is thought to promote healing. In a recent study, the human kidney cell death rate was found to be reduced by two-thirds when cultured in aloe vera gel.<sup>19</sup>

Low-fat milk also showed good clonogenic capacity. Its statistical significance was similar to the aloe vera extract. Blomlof et al.,<sup>20</sup> Hiltz et al.,<sup>13</sup> and Oikarinen et al.<sup>10</sup> showed better maintenance of PDLF vitality, proliferation, enzyme activity, and total protein synthesis including collagen *in vitro* which support our study. These favorable results probably occurred because milk contains important nutritional substances, such as amino acids, carbohydrates, and vitamins. In addition, commercially available milk is pasteurized, which may inactivate enzymes that could be potentially harmful to the PDLF. The suitability of milk as a storage medium is further emphasized by its general availability.<sup>11</sup>

However soy milk showed least proliferation. Moura et al.,<sup>21</sup> Silva et al.,<sup>22</sup> Moazami et al.<sup>23</sup> showed better cell viability with soy milk but clonogenic capacity was not studied. According to our knowledge, clonogenic capacity of soy milk has not been tested till now. Still further more number of studies with more samples is required to prove its clonogenic capacity.

The present study design presents some limitations. The *in vitro* culture of PDL cells does not simulate the oral environment. However, there is lack of adequate research in evaluating clonogenic capacity of HBSS, low-fat milk, soy milk, and aloe vera extract. Hence, we recommend future research on these storage medias.

This study concludes that the following:

- HBSS and aloe vera demonstrated highest clonogenic capacity at 24 hours.

- Low-fat milk also showed clonogenic capacity similar to aloe vera.
- However, soy milk demonstrated clonogenic capacity only at 2 hours of storage; thereafter, cell growth declined.

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