

**The efficacy of Green Tea Extract (GTE) as a medium for storage of Avulsed Tooth: An in-vitro study.**

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**Abstract**

**Introduction:** Green tea extract (GTE) is considered to show remarkable antioxidant, anti-inflammatory and anticarcinogenic effects and to prolong survival of allografts.

**Aim:** The present in vitro study was conducted to investigate the efficacy of green tea extract as a storage medium for avulsed teeth. We assessed the possibility for the selected storage medium by maintaining the viability of human periodontal ligament (PDL) cells.

**Materials and Method:** Human periodontal ligament cells were cultured and stored in the following media: (1) Hank’s balanced salt solution (HBSS), (2) tap water, (3) milk, (4) Green Tea Extract, and (5) commercial green tea. After first, third, sixth, twelfth, and twenty fourth hours,

cells in various media were analyzed under the optical microscope and their viabilities were analyzed with a nucleocounter and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The data were then statistically analyzed by analysis of variance tests with post analysis through the Duncan method ( $P < .05$ ).

**Results:** There was no difference in PDL cell viability between GTE and HBSS media, whereas GTE showed relatively higher cell viability than other media ( $P < .05$ ).

**Conclusions:** The present study thus showed that the efficacy of Green Tea Extract in maintaining the viability of human PDL cells is similar to that of HBSS and higher than that of milk. Therefore, GTE could be used as a suitable, alternative storage medium for avulsed teeth.

**Keywords:** Catechin, cell viability, green tea, green tea extract, nucleocounter, periodontal ligament cell, storage media, tooth avulsion

### **Introduction**

Avulsion of tooth is one of the main concerns in dental traumatology due to its status as a major dental injury. Avulsion of teeth is encountered in dental setups on a day to day basis. Immediate replantation of avulsed tooth is so far known as the best treatment options so far (1). For successful replantation, preservation of the vitality of periodontal ligament (PDL) cells attached to the root remains pivotal (2).

A 15-minutes delay in replantation of avulsed tooth causes PDL cell damage which in turn causes partial resorption of root. If this delay is up to half an hour or 30 minutes then such replantation is severely damaging to the viability of cells. And a further delay of 1 hour or 60 minutes in a dry condition results in necrosis of PDL cells that leads to extensive resorption of root. (3) To obtain the best prognosis, extra-alveolar time should be less than 5 minutes only. (4) Lack of dental awareness and negligence on part of patients' ward result in failure to replant avulsed tooth immediately. With sufficient knowledge available on social media and internet these days, patients and guardians have now become well informed about proper storage media for avulsed teeth. Dentists should deal with various storage media in which patients store their avulsed tooth. To the best of our knowledge, several types of media have been used for the storing avulsed teeth. Such good media for storage include saliva, milk, Hank's balanced salt solution (HBSS) etc. Besides other studied and tested storage media include egg white, powdered milk, Gatorade, and honey bee extract- propolis (5-6).

Camellia sinensis is the source of Green tea extract (GTE) known for its wide consumption across world population, second only to water (7). Catechin present in Green tea

extracts (GTEs) is one of the polyphenols from green tea. Catechins in GT are epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin-3-gallate (EGCG) etc (7). GTEs have shown remarkable antioxidant, anti-inflammatory, and anticarcinogenic effects for treating a number of animal tumors, cell culture systems, and epidemiological studies (7,8). A recent animal study concluded that the GTE in combination with low-dose cyclosporine A showed possible prolongation of survivals of allografts in mice (9). In dentistry, the usage of GTEs is still being studied. EGCG was known to prevent the alveolar bone resorption from periodontal diseases because it inhibited matrix metalloproteinase-9 (MMP-9) expression in osteoblasts and also inhibits formation of osteoclasts (10). The purpose of this study was in vitro evaluation of the possibility of GTE as a substitute for storage media for avulsed teeth. In addition, we estimated the PDL cell viability in different storage media during variant storage durations.

### **Materials and Methods**

The study proposal for harvest and use of human periodontal ligament cell was reviewed and approved by the Institutional Ethical Review Board.

#### **Selection of Test Materials and Preparation of GTE**

We tested four commercial GTs and GTEs before the main experiment. The osmolality and acidity of individual medium was tested twice with an automatic cryoscopic osmometer and a pH meter. One commercial GT and GTE were selected (Table 1). The GTE was made from the hot-water extract of GT for which ten grams leaves were soaked in 100 mL of distilled water boiled for 5 minutes then filtered and sterilized.

#### **Cell Culture of Human PDL Cells**

PDL cells were procured from clinically healthy sound premolars extracted for orthodontic purposes. Extraction was carried out as atraumatically as possible, and a tooth

was washed in sterile saline solution to get rid of any residual blood. A conical tube filled with HBSS was used for storing the tooth. The tooth was held with forceps from the coronal region, and the PDL cells were carefully obtained by scraping with a no. 11 scalpel blade from the lower two thirds of the root surfaces. The tissues were then sliced into small pieces and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

### 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 1: HBSS, (2) group 2: tap water, (3) group 3: milk, (4) group 4: commercial GT; and (5) group 5: GTE.

Table 1: The pH and Osmolality of Experimental Solutions

Group	pH	Osmolality (mosmol/kg)
HBSS	7.382	284
Tap water	7.134	3
Milk	6.709	288
GT	6.294	12
GTE	5.913	139

### Microscopic Examination and Assessing Cell Viability

#### Using a Nucleocounter

The 2X10<sup>5</sup> periodontal ligament cells were placed in six-well plates, followed by overnight incubation 37°C with 5% CO<sub>2</sub>. The culture medium was then removed from the well, and 2 mL of five different experimental solutions were added at room temperature.

Each well was examined under the optical microscope after 1, 3, 6, 12, and 24 hours, and then cell viability was analyzed using a nucleo-counter. NC-100 is basically a fluorescence microscope that counts cells automatically.

The integrated fluorescence microscope is designed to detect signals from the fluorescent dye, propidium iodide (PI), which is bound to cell nuclei. PI is immobilized in the interior of the disposable Nucleocassette. A light-emitting diode emits green light in order to excite the PI-DNA intercalation. The NC-100 counts either a total or a nonviable cell concentration.

After exposure in each medium, 100 mL of 0.25% trypsin was added to each medium, and the plates were incubated at 37°C for 5 to 10 minutes. The samples were scrapped with a scrapper and centrifuged for 4 minutes at 1,000 rpm. After centrifugation, the supernatant was removed, and the culture medium was added up to 200 mL.

To count total cell number, the 200-mL lysis buffer was added to the cell sample and thoroughly mixed by turning the vial upside down five to ten times. Then, the 200 mL of stabilizing buffer was also added and mixed thoroughly. The Nucleocassette with the stabilized mixture was loaded immediately after mixing and inserted to the NC-100. No pretreatment of the cell sample was needed to count the nonviable cells. The cell suspension was mixed to obtain a homogenous suspension, and the cells were loaded directly into the Nucleocassette.

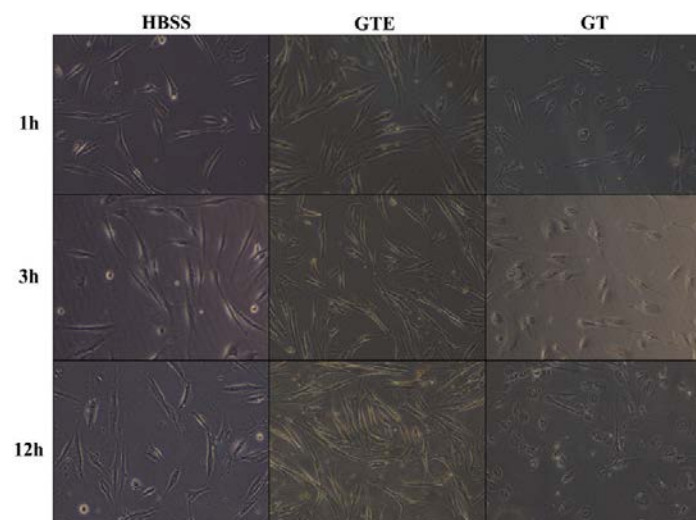


Figure 1: A microphotograph of PDL cells in HBSS, GTE, and GT at different storage durations.

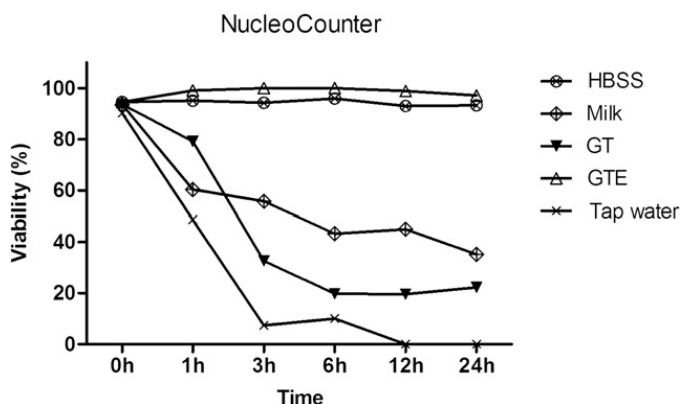


Figure 2: The viability of PDL cells maintained in each experimental medium.

### Statistical Analysis

We used SPSS for Windows evaluation version 18.0 was used in order to evaluate the viability rate according to variable storage hours. We estimated the parameters with the SPSS general linear model univariate procedure and two-way analysis of variance with 5% significance level. Each test was complemented by post analysis using Duncan’s method.

### Results

#### Microscopic Examination

Microscopic analysis showed that the PDL cells stored in the HBSS and GTE kept almost the spindle-like morphology as time passed. However, cells stored in the tap water could not maintain their own shape even after 3 hours (Fig. 1). In the microscopic examination, HBSS was effective in maintaining the viability of PDL cells. In the case of GTE, we could not find any signs of modification or destruction of cells. The cells maintained their shape for 24 hours and showed to maintain quite stable at room temperature. The cells in the GTE turned green. It might be due to the nature of GT. The cells in the tap water showed destruction of cell morphology in a low osmolality condition within an hour. Many cells were floating around as a result of cell death. Also, it was hard

to observe the cell shape in milk under the microscope because of its own color.

#### Cell Viability Test Using a Nucleocounter

After it had been stored in the five different media at room temperature, the cell viability was estimated by using a nucleocounter (Fig. 2). Among the five experimental groups, HBSS and GTE made over 90% of PDL cells alive for 24 hours, whereas only 19.1% of cells on average survived in the other media. Interestingly, cells in GTE (97.2%) showed a little higher viability than those in HBSS (93.3%) although both of them did not show significant differences in the degree ( $P < .05$ ) (Table 2). The cell viability in milk, GT, and tap water dropped quickly within 3 hours compared with those in HBSS and GTE. After 3 hours, the viability in milk was higher than those in GT and tap water. Interestingly, cells in GT were more viable than those in milk within an hour. By imaging through Nucleoview, a lot of nonviable cells were observed in milk, GT, and tap water increased when time passed, whereas nonviable cells were not observed in HBSS and GTE (Fig. 3).

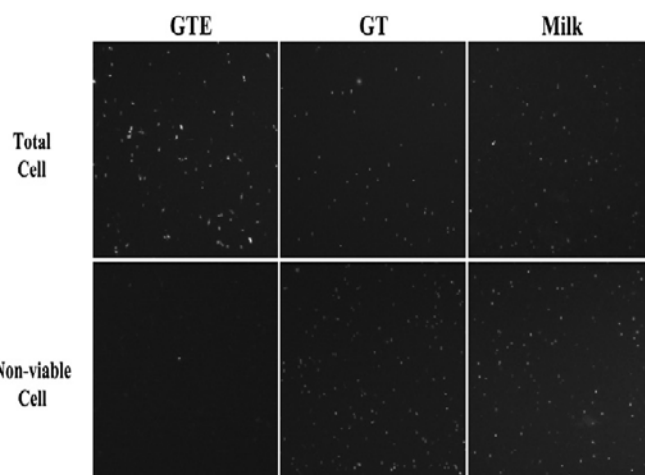


Figure 3: The image through the Nucleoview in GTE, GT, and milk at 24 hours.

TABLE 2. The Viability of Periodontal Ligament Cells at Each Experimental Medium Using a Nucleocounter (unit: %)

Storage time		0h	1h	3h	6h	12h	24h
HBSS	1	94.6 T1.5 <sup>a</sup> 93.3 T8.2 <sup>a</sup>	95.1 T3.2 <sup>a</sup>	94.3 T1.6 <sup>a</sup>	96.0 T1.8 <sup>a</sup>	92.9	T 2.9 <sup>a</sup>
Tap water	2	90.2 T6.0 <sup>a</sup> 0.0 T0.0 <sup>a</sup>	48.6 T11.8 <sup>c</sup>	7.4 T12.8 <sup>c</sup>	10.0 T17.3 <sup>c</sup>	0.0	T 0.0 <sup>a</sup>
Milk	3	93.6 T2.1 <sup>a</sup> 35.1 T1.8 <sup>b</sup>	60.4 T11.4 <sup>bc</sup>	55.8 T18.8 <sup>b</sup>	43.2 T4.3 <sup>b</sup>	45.0	T 11.6 <sup>b</sup>
GT	4	93.6 T2.9 <sup>a</sup> 22.2 T9.7 <sup>c</sup>	79.1 T18.1 <sup>ab</sup>	32.6 T24.1 <sup>bc</sup>	19.8 T7.8 <sup>c</sup>	19.6	T 4.2 <sup>c</sup>
GTE	1	94.6 T3.6 <sup>a</sup> 97.2 T4.9 <sup>a</sup>	99.0 T1.6 <sup>a</sup>	100.0 T0.0 <sup>a</sup>	100.0 T0.0 <sup>a</sup>	98.8	T 2.0 <sup>a</sup>

1, 2, 3, and 4 indicate significant differences in the two-way analysis of variance test (P < .05).

Different letters indicate statistical significant in one-way analysis of variance test comparing each group at the same storage time (P < .05).

### Cell Viability Test by MTS Assay

Cell viability was measured alternatively by using an MTS assay (Table 3). The OD of milk was significantly higher than those of other groups (P < .05). The results of other solutions had similar tendencies from the nucleocounter. There were no significant differences in the OD between HBSS and GTE (Table 3). At 1 hour, there were no significant differences among the three solutions (GTE, GT, and tap water; P < .05). However, after 3 hours, the GT group had a significantly lower OD (P < .05) than those of GTE and HBSS. In comparison to GT and tap water, there were no significant differences between those media although the OD value of GT was higher than that of tap water.

### Discussion

Extraoral time and storage medium are two important deciding factors for prognosis of avulsed tooth (8). The longer exposure of avulsed tooth to dry storage worsens the prognosis of replantation. Therefore, many studies did intend to preserve avulsed tooth in proper medium (7–9). The medium used for storage should be capable of preserving vitality of PDL cells and their adherence

capacity. Immediate availability of storage medium at the time of avulsion allows its rapid access (9). Both physiological osmolality and pH are crucial factors in preserving the viability of PDL cells. An osmolality of 230 to 400 mosmol/kg and a pH of 6.6 to 7.8 is necessary for the growth of cells mainly happen at (8).

In this study, HBSS, tap water, milk, commercial GT, and GTE were tested for the effect of maintaining the viability of PDL cells. HBSS is a widely used standard solution recommended by the International Association of Dental Traumatology as the best storage media for avulsed tooth. The osmolality and pH of HBSS are 270 to 290 mosmol/kg and 7.2, respectively. Although HBSS has the ability to provide long-term preservation of PDL cells, it is not yet available in pharmacies or drug stores at the scene of an accident. So, milk is known as the appropriate storage medium for avulsed tooth because it is easy to get in the event of an accident and able to maintain PDL cells. In addition, milk has a physiologically compatible pH and osmolality, many essential nutrients, and growth factors (9). However, tap water used as a negative control in this study, is ineffective in maintaining PDL cell viability because it leads to rapid death of PDL cells because of its hypotonic properties and high chances of bacterial contamination (5).

Storage time		0h	1h	3h	6h	12h	24h
HBSS	1	0.52 T0.06 <sup>a</sup> 0.34 T0.04 <sup>bc</sup>	0.42 T0.07 <sup>ab</sup>	0.351 T0.02 <sup>b</sup>	0.432 T0.10 <sup>b</sup>	0.401	T 0.067 <sup>a</sup>
Tap water	2	0.48 T0.09 <sup>a</sup> 0.25 T0.01 <sup>c</sup>	0.26 T0.03 <sup>a</sup>	0.25 T0.02 <sup>b</sup>	0.24 T0.03 <sup>c</sup>	0.25	T 0.03 <sup>c</sup>
Milk	3	0.502 T0.12 <sup>a</sup> 1.39 T0.13 <sup>a</sup>	0.512 T0.16 <sup>a</sup>	0.534 T0.23 <sup>a</sup>	0.632 T0.14 <sup>a</sup>	1.13	T 0.10 <sup>a</sup>
GT	2	0.481 T0.08 <sup>a</sup> 0.30 T0.01 <sup>c</sup>	0.312 T0.04 <sup>b</sup>	0.292 T0.03 <sup>b</sup>	0.283 T0.02 <sup>c</sup>	0.31	T 0.01 <sup>c</sup>
GTE	1	0.50 T0.05 <sup>a</sup> 0.42 T0.03 <sup>c</sup>	0.37 T0.04 <sup>b</sup>	0.38 T0.03 <sup>b</sup>	0.37 T0.05 <sup>bc</sup>	0.43	T 0.01 <sup>b</sup>

1, 2, and 3 indicate significant differences in the two-way analysis of variance test ( $P < .05$ ).

Different letters indicate a statistical significant in one-way analysis of variance test comparing each group at the same storage time ( $P < .05$ ).

The MTS assay has the same principle with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, where the amount of formed formazan by means of mitochondrial dehydrogenase activity is proportional to the number of living cells.

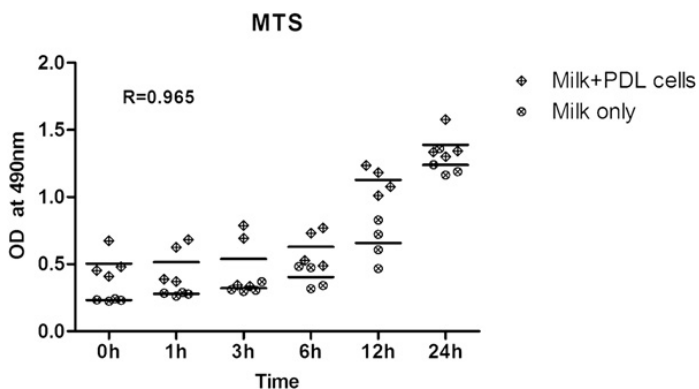


Figure 4: The MTS patterns in milk with/without PDL cells.

Our results clearly indicated that HBSS, GTE, and milk were effective storage media in maintaining viability of PDL cell. Especially, HBSS and GTE were most effective in preserving PDL cell viability for 24 hours because they could preserve the viability over 90%. The result of HBSS effectiveness is in agreement with other studies (8). GTE has enough catechin, which is an antioxidant-rich ingredient and thus is thought to be effective in maintaining PDL cell viability.

A recent study that used propolis as a storage medium for the avulsed tooth suggested that the higher viability of PDL cells might be because of the antibacterial and anti-inflammatory abilities of propolis (7). This finding may explain the reason cells in GTE exhibited a little higher viability than cells in HBSS. Along with GTE's potential to keep PDL cells more viable, perhaps future research

can show how its anti-bacterial and anti-inflammatory properties are effective in resorption sequelae that often lead to the loss of tooth after replantation.

### Conclusion

In conclusion, the prognosis of an avulsed tooth is mainly based on the viability of the PDL cells. Within the limit of this study, the efficacy of GTE in maintaining the viability of human PDL cells was similar to that of HBSS and better than that of milk. Therefore, GTE can be an alternative media for the storage of avulsed teeth in case of the absence of HBSS.

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