

The Use of Green Tea Extract as a Storage Medium for the Avulsed Tooth

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Abstract

Introduction: Green tea extract (GTE) has been reported to have remarkable anti-inflammatory, antioxidant, and anticarcinogenic effects and to prolong allograft survivals. The purpose of the present study is to investigate *in vitro* the efficacy of GTE as a storage medium for avulsed teeth. We estimated the possibility for storage medium by maintaining the viability of human periodontal ligament (PDL) cells. **Methods:** Human PDL cells were cultured and stored in the following media: (1) Hank's balanced salt solution (HBSS), (2) tap water, (3) milk, (4) GTE, and (5) commercial green tea. After 1, 3, 6, 12, and 24 hours, cells in different media were examined under the optical microscope, and their viabilities were analyzed by using a nucleocounter and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The data were statistically analyzed by analysis of variance tests with post analysis using the Duncan method ($P < .05$). **Results:** The result indicates that there was no difference in cell viability between GTE and HBSS media, whereas GTE showed higher cell viability than other media ($P < .05$). **Conclusions:** Our study shows that the efficacy of GTE in maintaining the viability of human PDL cells is similar to that of HBSS and higher than that of milk. Therefore, we conclude that GTE could be a suitable, alternative storage medium for avulsed teeth. (*J Endod* 2011;37:962–967)

Key Words

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

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Tooth avulsion is one of the main issues in dental traumatology because of its status as a severe dental injury. Because dentists commonly encounter a tooth avulsion in the dental clinic, immediate tooth replantation is widely known as the best clinical treatment (1, 2). For successful replantation, it is most important to preserve the vitality of periodontal ligament (PDL) cells attached to the root (3).

When an avulsed tooth is replanted after 15 minutes, damaged PDL cells cause partial root resorption. Moreover, a 30-minute delayed replantation can cause fatal damage to cells, and a 60-minute delayed replantation in a dry condition can cause PDL necrosis, leading to extensive root resorption (3–5). The best prognoses of tooth replantation are obtained when the extra-alveolar time does not exceed 5 minutes (6). However, the lack of specific dental knowledge may lead a patient and his/her guardian to fail to replant an avulsed tooth in a timely manner.

With abundant information available in the media and also on the Internet nowadays, the patient and guardian should be well aware that the avulsed tooth must be kept in a proper storage medium. Dentists must deal with various storage media in which patients store their avulsed tooth. To this day, several types of media have been used for the storage of avulsed teeth. Good examples are saliva, milk, Hank's balanced salt solution (HBSS), Save-A-Tooth system (Phoenix-Lazerus, Shartlesville, PA) and ViaSpan (DuPont Pharmaceuticals, Wilmington, DE). Other storage media including egg white, powdered milk, Gatorade (The Gatorade Co, Chicago, IL), and propolis are being recently studied and tested (7–9).

Green tea (GT), extracted from *Camellia sinensis*, is a widely consumed beverage throughout the world that is second only to water (10). Green tea extracts (GTEs) contain catechin, which is one of the polyphenols from GT. Catechins in GT are catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin-3-gallate (EGCG), and so forth (11). It has been reported that GTEs have remarkable anti-inflammatory, antioxidant, and anticarcinogenic effects in a number of animal tumors, cell culture systems, and epidemiological studies (12, 13). A recent animal study showed that the GTE in combination with low-dose cyclosporine A prolonged allograft survivals in mice (14).

In the dental field, the usage of GTEs has been still studied. EGCG was known to protect the alveolar bone resorption from periodontal diseases because it inhibited the expression of matrix metalloproteinase-9 (MMP-9) in osteoblasts and the formation of osteoclasts (15). Also, EGCG suppressed the progression of apical periodontitis, possibly by diminishing osteoblastic expression of cysteine-rich 61 and subsequently macrophage chemotaxis into the lesions in rats (16). A recent study showed that GT had an antimicrobial effect as a root canal irrigant (17).

The purpose of this research was to evaluate *in vitro* 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

This study was performed at Kyung Hee University, School of Dentistry, Seoul, South Korea. The research proposal for harvest and use of human periodontal ligament cell was reviewed and approved by the Institutional Review Board of the Kyung Hee Medical Center, Kyung Hee University (KHD-IRB: 2009-8).

Selection of Test Materials and Preparation of GTE

We tested four commercial GTs and GTEs before the main experiment. The osmolality and acidity of each media was tested twice with an automatic cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany) and a pH meter (HI 83141; Hanna Instruments, Ann Arbor, MI). We selected one commercial GT and GTE (Table 1). The GTE was prepared from the hot-water extract of GT (Boseong, Gwangju, Korea); 10 g of GT leaves were soaked in 100 mL of boiling distilled water for 5 minutes and filter sterilized. This study applied the same methodology used by previous studies (18, 19).

Cell Culture of Human PDL Cells

PDL 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 lower two thirds of the root surfaces. The tissues were split into small pieces and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL) in a humidified atmosphere containing 5% CO₂ 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 (passages 3–4 were used).

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 (Seoul Milk; Seoul Milk Co, Gyeonggi-do, Korea), (4) group 4: commercial GT (Bosung Green Tea; Dongwon F&B, Seoul, Korea); and (5) group 5: GTE (Boseong, Gwangju, Korea).

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 2 × 10⁵ PDL cells were plated in six-well plates, and these plates were incubated at 37°C with 5% CO₂ overnight. The culture medium was 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 nucleocounter (NC-100; Chemometec, Allerød, Denmark). 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 iodine (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 μL 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 μL.

To count total cell number, the 200-μL lysis buffer was added to the cell sample and thoroughly mixed by turning the vial upside down five to ten times. Then, the 200 μL 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.

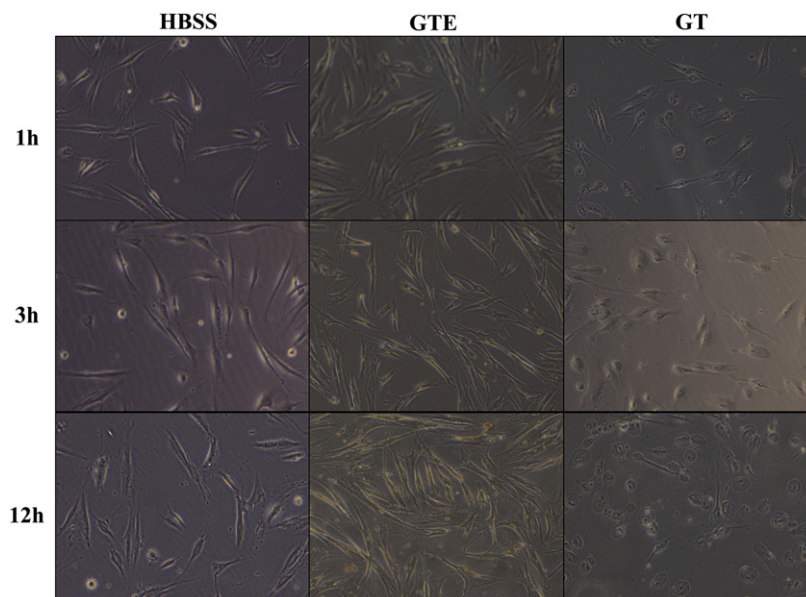


Figure 1. A microphotograph of PDL cells in HBSS, GTE, and GT at different storage durations.

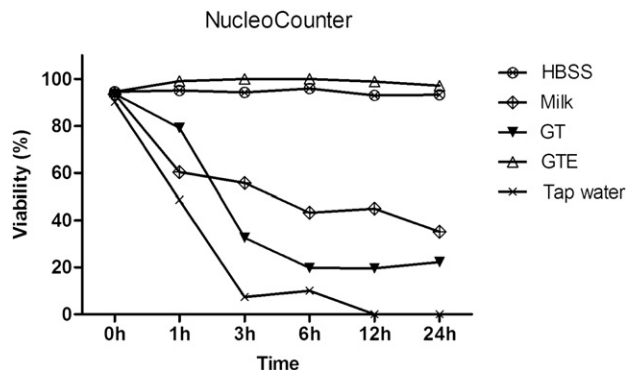


Figure 2. The viability of PDL cells maintained in each experimental medium.

When both the concentration of nonviable cells and the total concentration of cells are known, it is possible to calculate the viability rate (%) of the cells. The calculation can be performed by the use of Nucleoview (Chemometec; Windows 2000, version 2.2).

Cell Viability by the 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulphophenyl)-2H-Tetrazolium Assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega Co, Madison, WI). For each experiment, 5×10^3 cells in supplemented DMEM were plated in 96-well tissue-culture plates and incubated at 37°C in 5% CO₂ and 95% air for 24 hours. Subsequently, the medium was removed, and 200 μL of each of the different experimental solutions was added. The plates ($n = 5$) were maintained at room temperature for 0, 1, 3, 6, 12, and 24 hours. After the appropriate time, the MTS solution (20 μL/mL) in DMEM medium was placed in each well, and the plates were incubated at 37°C in 5% CO₂ and 95% air for additional 2 hours. To quantify the viability of metabolically active cells, the optical density (OD) of the solubilized formazan product was measured by means of the enzyme-linked immunosorbent assay at a 490-nm wavelength.

Statistical Analysis

We used SPSS for Windows evaluation version release 15.0.0 (September 6, 2006; SPSS Inc, Chicago, IL) 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. When this method revealed the interaction between any of the primary factors, one-way analysis of variance and a post analysis by Duncan's method were used to detect statistically significant differences among different storage durations in the same media.

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).

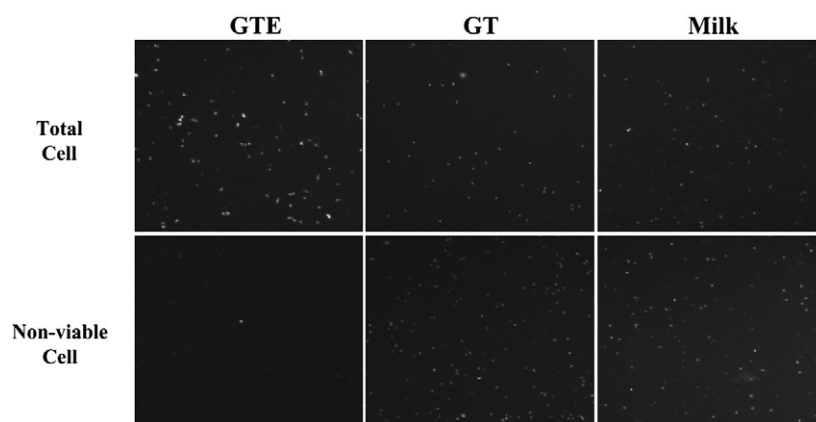


Figure 3. The image through the Nucleoview in GTE, GT, and milk at 24 hours.

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

Experimental groups		Storage time					
		0 h	1 h	3 h	6 h	12 h	24 h
HBSS	1	94.6 ± 1.5 ^a	95.1 ± 3.2 ^a	94.3 ± 1.6 ^a	96.0 ± 1.8 ^a	92.9 ± 2.9 ^a	93.3 ± 8.2 ^a
Tap water	2	90.2 ± 6.0 ^a	48.6 ± 11.8 ^c	7.4 ± 12.8 ^c	10.0 ± 17.3 ^c	0.0 ± 0.0 ^d	0.0 ± 0.0 ^d
Milk	3	93.6 ± 2.1 ^a	60.4 ± 11.4 ^{bc}	55.8 ± 18.8 ^b	43.2 ± 4.3 ^b	45.0 ± 11.6 ^b	35.1 ± 1.8 ^b
GT	4	93.6 ± 2.9 ^a	79.1 ± 18.1 ^{ab}	32.6 ± 24.1 ^{bc}	19.8 ± 7.8 ^c	19.6 ± 4.2 ^c	22.2 ± 9.7 ^c
GTE	1	94.6 ± 3.6 ^a	99.0 ± 1.6 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	98.8 ± 2.0 ^a	97.2 ± 4.9 ^a

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 the critical factors responsible for prognosis of avulsed tooth (8, 20, 21). The longer the exposure of avulsed tooth to dry storage, the worse the prognosis of explantation is. Therefore, many studies aimed to preserve avulsed tooth in proper medium (7–9, 21–23). The storage medium should be capable of preserving cell vitality and adherence capacity and also be readily available at the moment of avulsion to allow its rapid access (9, 22). Both physiological osmolality and pH are important factors in preserving the viability of PDL cells. It has been reported that the growth of cells mainly happen at an osmolality of 230 to 400 mosmol/kg and a pH of 6.6 to 7.8 (8, 23).

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 a storage medium for avulsed tooth (24). 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 (23), 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, 22). However, tap water, which was 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 incidence of bacterial contamination (5, 23).

The commercial GT used in this study showed very low osmolality, which might lead to PDL cell death. However, GT was chosen as a test solution because people in East Asia generally drink GT and it is easily available in the event of an accident. GTE was selected as a test medium because previous studies used GTE as a solution for allograft and cell study (18, 19). Interestingly, GTE showed the best ability for storage of an avulsed tooth although the osmolality and pH of GTE was not ideal.

In the previous studies, various techniques were used to quantitate the number of viable PDL cells including the stepwise trypsinization procedure (25), used chromogenic stain (4), and the stepwise trypsinization procedure and fluorescein diacetate (26). Recently, Chamorro et al (22) introduced a terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling (TUNEL) technique for testing the Apop-Tag Fluorescein *In Situ* Apoptosis Detection Kit (CHEMICON International Inc, Temecula, CA). Among those methods, the hemocytometer and trypan blue exclusion method is the most commonly used to test for cell viability (27). This method has been also used widely in the industry because it is cheap and easy. However, it seems time-consuming, labor intensive, and subjective in the determination of cell numbers (28).

On the other hand, the NC-100 used in this study provided faster, more efficient, and more reliable values than the traditional hemocytometer and trypan blue exclusion method. This device could reduce intra- and interobserver variations as well as provide consistency in repetitive analysis. The main advantages of this technique are the ability to handle a large number of samples with a high degree of precision and its simplicity and specificity in detecting viable cells quantitatively (28). In contrast, the disadvantage of NC-100 is the inability to measure the number of cells if it is below 5×10^3 . Therefore, as recommended by the manufacturer, the number of 10^5 cells was seeded, and at the same time, regarding the possibility of an interference caused by cell density, a six-well plate was used.

In addition, the MTS assay was used to complement the limitations of quantitative analysis. The MTS assay has the same principle with the 3-(4,5-

TABLE 3. Optical Density of Each Experimental Media

Experimental groups		Storage time					
		0 h	1 h	3 h	6 h	12 h	24 h
HBSS	1	0.51 ± 0.06 ^a	0.41 ± 0.07 ^{ab}	0.35 ± 0.02 ^b	0.43 ± 0.10 ^b	0.40 ± 0.07 ^b	0.34 ± 0.04 ^{bc}
Tap water	2	0.48 ± 0.09 ^a	0.26 ± 0.03 ^a	0.25 ± 0.02 ^b	0.24 ± 0.03 ^c	0.25 ± 0.03 ^c	0.25 ± 0.01 ^c
Milk	3	0.50 ± 0.12 ^a	0.52 ± 0.16 ^a	0.54 ± 0.23 ^a	0.63 ± 0.14 ^a	1.13 ± 0.10 ^a	1.39 ± 0.13 ^a
GT	2	0.48 ± 0.08 ^a	0.31 ± 0.04 ^b	0.29 ± 0.03 ^b	0.28 ± 0.02 ^c	0.31 ± 0.01 ^c	0.30 ± 0.01 ^c
GTE	1	0.50 ± 0.05 ^a	0.37 ± 0.04 ^b	0.38 ± 0.03 ^b	0.37 ± 0.05 ^{bc}	0.43 ± 0.01 ^b	0.42 ± 0.03 ^b

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 ($P < .05$).

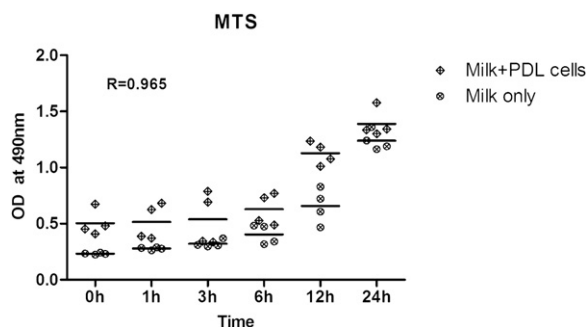


Figure 4. The MTS patterns in milk with/without PDL cells.

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. The metabolic activity of the cells we measured can be evidence for the estimation of the metabolic state of the surviving cells, with other results of viable cells (29). Unlike formazan formed from the MTT assay, formazan formed from the MTS assay has a high solubility that does not need organic solvents. Therefore, the cells in the process of medium elimination have no risk of damage, and compared with the existing MTT assay, a high level of sensitivity is characterized by visibility (30, 31).

Our results indicated that HBSS, GTE, and milk were effective in maintaining PDL cell viability. 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, 21, 32). Buttke and Trope (33) suggested that the storage of avulsed teeth in a medium containing one or more antioxidant components might increase the success rate of replantation. *Salvia officinalis*, an herb similar to GT, was found to be an appropriate storage solution for the dislocation of teeth because of the nature of the antioxidant activity (32). GTE has enough catechin, which is an antioxidant-rich ingredient and thus is thought to be effective in maintaining PDL cell viability. Commercial GT was a less effective solution than the other three solutions mentioned previously because it showed a hypotonic property and contained other chemical tastes. However, GT would be seen as being more effective than tap water and, in the limit of our results, during the short period of 1 hour, GT is considered to be available as storage media of avulsed teeth.

The MTS assay showed a similar tendency to those of NC-100 except milk. The OD of the milk in the MTS assay was significantly higher than any other solution and increased as time elapsed. The previous study using MTT assay also reported that PDL cells stored in milk had a higher OD than that in HBSS (23). These results might be associated with the growth of bacteria because we did not sterilize the milk. Because metabolic activity in the presence of bacteria in the milk occurs, the high OD compared with other test solutions is displayed. The second cause was the opaque color of the milk itself. Smees et al (34) found that the colored substances affected the actual measured absorbance so that the value could be higher than stated. Thus, because of the unique color of milk, observing the absorbance is expected to have errors.

To confirm this hypothesis for the high OD value of milk, we compared the OD values of milk itself and milk with PDL cells (Fig. 4). Correlation analysis of the two groups showed a very high correlation coefficient ($r = 0.965$) over time, indicating that such a high tendency of milk in the MTS assay was the result of its own property.

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 antibacterial and anti-inflammatory properties are effective in resorption sequelae that often lead to the loss of tooth after replantation. Therefore, additional animal experiments to observe histological changes are thought to be worthwhile.

In conclusion, the prognosis of an avulsed tooth is mainly based on the status 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.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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