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Evaluation of the Viability of Rat Periodontal Ligament Cells after Storing at 0°C/2 MPa Condition up to One Week: *In Vivo* MTT Method

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Purpose: The aim of this study was to evaluate the rat periodontal ligament cell viability under 0°C/2 MPa condition up to one week using *in vivo* 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay.
Materials and Methods: As soon as 110 upper molar teeth of rats were extracted, they were stored in Hartman's solution under 0°C/2 MPa condition for 1, 2, 3, 4, and 7 days each. All specimens were treated with *in vivo* MTT assay and the value of optical density was measured by ELISA reader. These values were statistically analyzed by one-way ANOVA.
Result: There was no statistical difference on MTT value between immediate and 1 day storage group. There were statistically significant differences between 1 day and 2 days storage, 2 and 3 days storage groups, respectively. Teeth of 3, 4, and 7 days storage groups showed significantly lower MTT values compared with shorter period storage groups.
Conclusion: When the MTT values were substituted in standard curve, 1 day storage group at 0°C/2 MPa condition showed 68% cell viability when compared with immediate group. It dropped to 13% at 2 days, and to less than 5% at 3 days or more.

Key Words: MTT assay; Period; Periodontal ligament cell; Tooth storage

Introduction

In the tooth replantation procedure, it is most important to maintain the soundness of the periodontal ligament (PDL) cells on the root surface[1-3]. Maintaining the PDL cells on the root surface is critical[4,5] because the viability of the PDL cell is closely connected with an extracted tooth's extra oral time[6]. For delayed tooth replantation, the need for storage of extracted tooth had been

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increasing. Various researches have studied the ideal condition for tooth storage because the PDL cells viability could alter depending on the method of storage[7-11].

In the study of Lee[12] on the ideal condition for maintaining the activity of the PDL cells, the best result was obtained under 0°C/2 MPa during 2 days of storage. Despite these efforts to find the ideal tooth storage conditions, few studies on the duration of the high cell viability have been conducted.

The most frequently used method of evaluating the viability of the PDL cells is counting the number of cells collected from the root[9]. This method is easy and convenient because the cells, not the tissues, are counted. In this method, however, not all the cells on the tooth root surface are collected, and only about 70% of the living cells can divide, thereby restricting the healing capability of the periodontal tissues[13]. In addition, the number of cultured cells collected from the tooth root surface may differ from the number of the primary cells[14]. Accordingly, methods of easily and objectively evaluating a large amount of tissues are needed. The 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay that Mosmann[15] introduced was used in the studies on cell toxicity or survival capability[16]. Henze et al.[17] measured the depth of a burn wound using the tissues and not the cells through *in vivo* MTT assay to investigate cell activity in the tissues after making a wound on the pig skin, processing it with modified MTT staining, preparing frozen samples, and observing them under a microscope. Kim et al.[9] also histologically observed and compared the cell activities in the immediate MTT assay group and the MTT assay group placed under 1-hour dry storage after tooth extraction. The results of *in vivo* MTT assay and the histologic observation coincided. However, there were no studies about the period for tooth storage at the ideal condition. Therefore, the aim of this study was to evaluate the viability of the PDL cells using *in vivo* MTT assay, when stored at 0°C/2 MPa condition up to one week.

Materials and Methods

1. Standard Curve of MTT Assay

A standard curve was required to understand the relationship between the optical density values obtained from the MTT assay and the cell numbers, and to investigate the cell reduction with the lapse of time. Accordingly, the MTT assay was performed using the NIH3T3 cells. First, the NIH3T3 cells were cultured in media that contained Dulbeco's modified Eagle's medium (DMEM; Gibco®, Carlsbad, CA, USA) and fetal bovine serum (FBS; Gibco®) so that they could be counted. Then each cell was reacted with 200 µl of an MTT solution at 37°C for 2 hours and then with 1 ml of a dimethyl sulfoxide (DMSO; Ameresco Inc., Solon, OH, USA) solution for 15 minutes. After the cells settled down, they were separated from the solution, and the 200 µl DMSO solution was moved to the 96-well plate. The plate was put into microplate reader (Dynatech MRX ELISA microplate reader; Dynatech Laboratories, Chantilly, VA, USA) to measure the optical density at the 570 nm wavelength. The changes in the optical density according to the cell number are shown in Fig. 1.

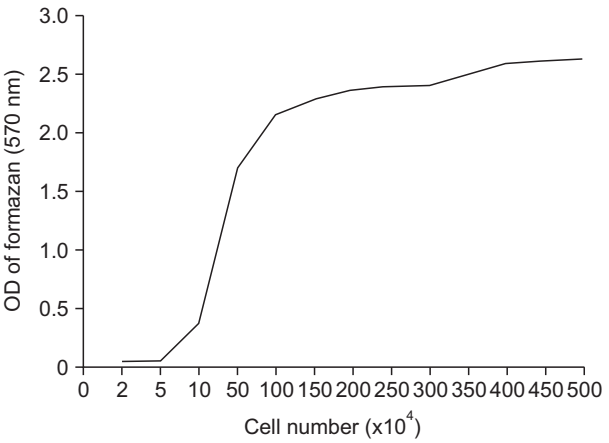

Fig. 1. Standard curve of the 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay using NIH3T3 cells. OD: optical density.

2. *In Vivo MTT Assay of Extracted Teeth*

Twenty-eight white female Spraque-Dawley rats weighing 100 g were anesthetized by injecting a mixture of tiletamine (20~40 mg/kg, Zoletil50; Virbac, Caroos, France) and xylazine (5~10 mg/kg; Rompun, Bayer, Germany) into its abdominal cavity. Peritomy was performed around the first and second upper molar on right and left per one rat using a sharp explorer before the tooth was extracted with a minimal wound in the surrounding tissues. Totally 112 rat teeth were extracted and only 110 teeth were used for experiments. All the roots were completely extracted without a fracture and washed by phosphate buffered saline (PBS; Gibco®) and then divided them by 11 groups (Table 1). Therefore 10 rat teeth were used in each group. Instantaneously implementing MTT assay was recognized as the immediate group and the negative groups included the teeth dried under root temperature. Storage groups were achieved based on the teeth kept for 1, 2, 3, 4, and 7 days under 0°C/2 MPa condition. The teeth were stored in a sealing bag filled with the Hartmann's solution (JW Pharmaceutical, Seoul, Korea) in a high hydrostatic pressure freezer (Dimapuretech, Bucheon, Korea). All groups are represented in Table 1. This experimentation is approved by Yonsei University Institutional Animal Care and Use Committee (2014-0114).

Table 1. Control group and experimental groups

Day	Group	Condition
	Immediate	
1	Dry	
	Storage	Room temperature: 0°C, 2 MPa
2	Dry	
	Storage	Room temperature: 0°C, 2 MPa
3	Dry	
	Storage	Room temperature: 0°C, 2 MPa
4	Dry	
	Storage	Room temperature: 0°C, 2 MPa
7	Dry	
	Storage	Room temperature: 0°C, 2 MPa

After 200 µl of a yellow MTT solution (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) was added into the 96-well plate, each group's teeth were put into each well that contained the MTT solution. To minimize the presence of blood cells, the tooth root surface was cleansed with a saline solution. The 96-well plate was wrapped with aluminum foil and cultured at 37°C for 3 hours to reduce the MTT solution. The teeth in each well were moved to another 96-well plate to soak them in 150 µl of DMSO (Ameresco Inc.) for 15 minutes. The teeth were then removed from each well and the plate placed in microplate reader to measure their optical density at a wavelength of 570 nm.

3. *Eosin Staining of In Vivo MTT Assayed Teeth*

To prevent variations in the amount of tissues on the surface of the extracted tooth roots and the consequent difference in the cell numbers of the groups, the cells were stained with eosin. The teeth stained with the MTT solution were soaked in a 350 µl eosin solution (Sigma-Aldrich Co., St. Louis, MO, USA) for 12 hours for further staining. They were bleached in 350 µl of 1% acid alcohol (1% hydro acid and 70% ethyl alcohol) for 15 minutes and removed from each well. The plate was put in microplate to measure the optical density at a wavelength of 530 nm. Differences in the cell numbers of the roots were corrected by dividing the optical density in the MTT assay by the optical density in the eosin-staining method.

4. *Statistical Analysis*

The optical density obtained from the MTT assay was divided by the optical density obtained from the eosin staining. The MTT/eosin values were statistically calculated using one-way ANOVA. For the post hoc analysis, the Bonferroni method was used. The significance level was 95%. All the statistical analysis was performed using IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA).

Result

The standard curve of the optical densities of the NIH3T3 cells obtained from the MTT assay is shown in Fig. 1.

With the increase in cell number, the optical density level also increased. Major changes in the optical density from the MTT assay were observed in the NIH3T3 cell number range of 5~100×10^4. At 100×10^4 cell number, the optical density was 2.169 and then no significant change in the optical density was observed with the increase in cell number from 100×10^4 cell number ($P<0.05$). The mean optical densities obtained from the MTT assay and the eosin staining are shown in Tables 2, 3 and Fig. 2.

Table 2. Optical density from the MTT assay and eosin staining method in storage group

Day	MTT	Eosin	MTT/eosin
Immediate	2.571±0.281	0.339±0.062	7.767±1.318[a]
1	2.419±0.151	0.352±0.068	7.087±1.392[a]
2	1.674±0.114	0.345±0.055	4.993±1.020[b]
3	0.476±0.099	0.349±0.072	1.365±0.446[c]
4	0.258±0.054	0.334±0.051	0.783±0.214[c]
7	0.128±0.051	0.253±0.068	0.534±0.301[c]

MTT: 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide.
Values are presented as mean±standard deviation.
Different superscript letters represent statistically significant difference by Bonferroni post hoc ($P<0.05$).

Table 3. Optical density of MTT assay and eosin staining method in dry group

Day	MTT	Eosin	MTT/eosin
1	1.355±0.233	0.346±0.084	4.058±0.957[a]
2	0.863±0.092	0.359±0.082	2.515±0.630[b]
3	0.367±0.107	0.340±0.069	1.085±0.284[c]
4	0.170±0.114	0.346±0.062	0.501±0.335[c]
7	0.106±0.031	0.317±0.100	0.352±0.124[c]

MTT: 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide.
Values are presented as mean±standard deviation.
Different superscript letters represent statistically significant difference by Bonferroni post hoc ($P<0.05$).

The activity of the PDL cells of the rat teeth stored at 0°C/2 MPa abruptly decreased when they were stored for three or more days (Fig. 2).

In all the groups, there was no statistically significant difference in the optical density levels in the eosin staining. The immediate group showed the highest cell viability with 7.767±1.318 of MTT/eosin. The MTT/eosin values of the immediate and 1-day storage groups did not show

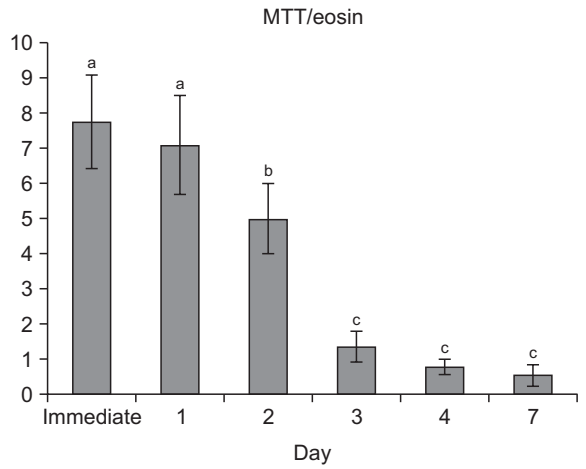

Fig. 2. MTT/eosin values of the immediate and storage groups. The error bars represent standard deviations (n=10). The different letters above the bars represent statistically significant difference between groups ($P<0.05$). MTT: 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide.

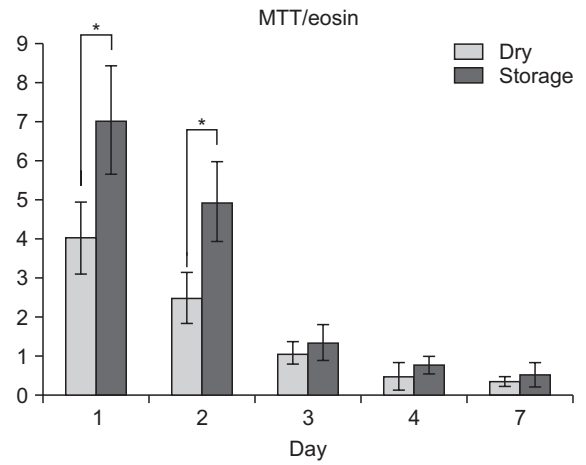

Fig. 3. Statistical comparison of MTT/eosin values between storage and dry groups. The error bars represent standard deviations (n=10). Asterisk above the bars represents statistically significant difference between groups ($P<0.05$). MTT: 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide.

a statistically significant difference (P>0.05; Table 2, Fig. 2). Between 1- and 2-day storage groups, and between 2- and 3-day storage groups, a statistically significant difference was observed (P<0.05); whereas between 3- and 4-day storage groups, and between 4- and 7-day storage groups, no significant difference was observed (P>0.05; Table 2, Fig. 2). At 0°C/2 MPa condition and under different storage durations, the storage group and the dry group did not show a statistically significant difference from 3 days (Fig. 3).

Discussion

The MTT assay used in this study for evaluating of cell viability reflects metabolically-active cell concentration levels by measuring the optical density values at the 570 nm wavelength using the process of reducing the yellow water-soluble tetrazolium salt (MTT; Sigma Chemical Co.) to a violet non-soluble MTT formazan crystal through the yellow dehydrogenized enzyme in the mitochondria of metabolically normal[15]. Therefore, we could realize the cell viability from the MTT solution color change and the value of optical density.

This study stored rat teeth under 0°C/2 MPa condition, which maintained the PDL cell higher in previous studies. Many studies presented that keeping the cell under pressure option maintained its viability higher than using organs such as tissues or embryos[18-21]. However, among the studies using teeth, the cell viability reduced rapidly over 3 MPa[12,19]. Therefore, this study selected the 0°C/2 MPa condition which represented consistently high cell viability. The Hartmann's solution that was used for tooth storage in this study can provide cells with extracellular fluid and correct metabolic acidosis. According to Lee[12], the Hartmann's solution and cellular fluid have electrolytes, so the freezing point of the Hartmann's solution and the rat PDL cells are lower than that of water. Accordingly, the cell viability can be kept high when the teeth are stored in the Hartmann's solution at an appropriate level of pressure and temperature. Lee[12] reported that the cells were not frozen while the teeth were stored.

While a rat tooth is small enough to be submerged in the well that it is sufficient for MTT assay, differences in number of cells on the root surface could be problematic. When the extracted maxillary first molar and second molar were directly used in the MTT assay, the number of PDL cells on the root surface are different. Even if the research was conducted on the same tooth, its root length and diameter are also different. Thus, a further investigation in number of cells on the root surface needs to be simultaneously undertaken[9], Jeon et al.[22] implemented H&E staining method to normalize the number of PDL cells. The MTT assay was applied in his study to measure the optical density (OD) value of solution of which H&E bleached by 1% acid alcohol. The problem, thus, could be solved with using the normalized ratio of MTT reduction OD value to the H&E OD value. There existed statistical significance between the maxillary first molar (1.067) and second molar (0.790) (P<0.05). And the maxillary first molar demonstrated the greater OD value. However, there was no statistical significance between groups using the same molar. When the H&E were stained with alkaline and acid stain solution, respectively, the hematoxylin stained the cell nucleus only. Therefore, to stain the cytoplasm or other structures, contrast staining should be conducted with eosin again. Furthermore, the OD values observed at 530 nm or 590 nm were different that examining two staining with an identical wavelength could be inappropriate[23]. Rather than that, studying on staining with eosin only was recommended as single staining, single wavelength analyses were more appropriate[22]. By applying the MTT assay, the amount of PDL cells could be indirectly demonstrated by eosin solution and their OD

value measured at 530 nm wavelength. Using the normalized number evaluated from dividing the MTT assay OD value by eosin staining OD value, molars having different amount of periodontal tissue could be converted to PDL unit area so they were comparable[12,18].

From the results, no significant difference in the optical density values of the immediate, dry, and storage groups from eosin staining was observed. This implies satisfactory extractions of all the teeth, and that the long-term tooth storage and drying at room temperature did not cause significant differences in the stained tooth surfaces.

The MTT/eosin values of the 1-day storage and immediate group did not show a statistically significant difference ($P>0.05$; Table 2, Fig. 2). This means the storage of teeth at 0°C/2 MPa resulted in PDL cells viability similar to that of the immediate group up to 1 day after teeth extraction. This can be confirmed in the standard curve, in which the 1-day storage group's MTT assay optical density value was 2.419±0.151 (270×10^4), which translated to an about 32% lower cell number than that with the immediate group's MTT assay optical density value of 2.571±0.281 (398×10^4). However, considering the almost constant curve, no significant difference between the two groups was confirmed. According to the standard curve, the formazan formation and the optical density values increased with the increase in cell number; but when the cell number reached 100×10^4 or more, the optical density values did not proportionally increase. This represents a limit that MTT solution can work at a certain concentration. Therefore, comparison between MTT assay results and standard curve is necessary for this type of experiment.

With the lapse of time, the PDL cells viability gradually declined. No statistically significant difference was observed among 3-, 4-, and 7-day storage groups, unlike between 1- and 2- day storage groups and between 2- and 3-day storage groups. Accordingly, storage at 0°C/2 MPa resulted in a highly maintained PDL cells viability for up to 2 days, but the viability significantly declined from 3 days. When the optical density values from the MTT assay were used in the standard curve, the 2-day storage group showed an about 87% decline in cell activity, unlike in the immediate group, and the 3-day storage group, about 95%. This means that for up to 2 days, the cell viability was highly maintained, but the number of cells was reduced by more than half.

The MTT/eosin values of the dry group also decreased with the lapse of time, and 1- and 2-day dry groups showed a statistically significant difference from the 1- and 2-day storage groups. These outcomes do not correspond to those of previous studies that reported that the PDL cells lost their differentiation and proliferation capabilities when they were exposed to a dry environment. This tendency was caused by the reduced functions of the mitochondria that produced adenosine triphosphate (ATP) as an energy source under the dry environment due to hypoxia[24,25]. Hypoxia is a signal of cell death that induces excessive movement of calcium ions via the permeability transition pore, which is a calcium channel, in the mitochondrial cell membrane. Consequently, ATP production is inhibited, and eventually, the cells reach an apoptosis condition[26]. However, according to Amemiya et al.[24], cell proliferation and differentiation increase at the early stage of apoptosis, and when the cells are in a hypoxic condition, their mitochondrial functions are enhanced to maintain their homeostasis and to improve the cell proliferation capability. In their experiments, the proliferation capability of the PDL cells increased for up to 3 days in the hypoxic condition. Therefore, the viability value approached zero after 1 day in the MTT assay because of the improvement in the mitochondrial functions for maintaining cell homeostasis in the early dry condition. The PDL cell viability can be confirmed through an MTT assay, but the optical density

value does not necessarily indicate the cell activity. Although the activity level can be determined, the level at which the cells can survive and function, and the appropriate level for tooth replantation, are not yet fully understood. In the completely dried cases, no group showed a zero level. This might be interpreted as maintenance of the cell activity despite the drying of the teeth. Further studies on details of the cell activity may be needed in the future. Although the limitation exist, this study could be a reference or standard of tooth storage period and the PDL cell vitality remaining period in replant surgery.

Conclusion

When the tooth was stored for 1 day at 0°C/2 MPa condition, high level of viability of the PDL cells could be sustained up to 68%. This subsequently dropped to 13% at 2 days, and to less than 5% at 3 days or more. From this result, the viability of the PDL cells could be maintained high up to 1-day storage and declined rapidly from 2-days storage at 0°C/2 MPa condition. Finally, our study could suggest the standard for tooth storage period for dental replantation, of which the extracted tooth was stored under low temperature and high pressure condition such as 0°C, 2 MPa.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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