ORIGINAL ARTICLE

J Korean Dent Sci. 2015;8(2):57-64
http://dx.doi.org/10.5856/JKDS.2015.8.2.57
ISSN 2005-4742

Effects of Slow Programmable Cryopreservation on Preserving Viability of the Cultured Periodontal Ligament Cells from Human Impacted Third Molar

Jin-Woo Kim[1,4], Tae-Yi Kim[4], Ye-mi Kim[2,4], Eun-Kyoung Pang[3,4], Sun-Jong Kim[1,4]

Departments of [1]Oral and Maxillofacial Surgery, [2]Conservative Dentistry, and [3]Periodontology, Ewha Womans University School of Medicine, [4]Graduate School of Clinical Dentistry, Ewha Womans University, Seoul, Korea

Purpose: This study was conducted to determine cell viability and differentiation capability of human periodontal ligament (PDL) cells and to elucidate the effects of cryopreservation on the activity of human third molar PDL cells by comparing PDL cells with and without cryopreservation.

Materials and Methods: Human PDL fibroblasts obtained from immature third molars were cultured and divided into two groups. The experimental group was cryopreserved with a slow freezing rate of 0.5°C/min from 4°C to –35°C followed by plunging in liquid nitrogen at –196°C and cultured after fast thawing. The control group was cultured without cryopreservation. Cell viability, growth capacity and morphology were evaluated in both groups. Bivariate statistics were used to compare 2 groups and linear mixed model analysis was used to investigate the growth trends difference over time.

Result: Cell viability and growth capacity were not significantly different between the 2 groups ($P>0.05$). Cultured cell of both groups showed fibroblast-like in appearance, and there were no significant differences in morphology between 2 groups. The mixed model analysis revealed no significant difference of growth capacity between 2 groups over time ($\beta=-0.0009$; $P=0.138$).

Conclusion: This study demonstrates that cryopreservation under control does not affect the biological properties of PDL cells, supporting the feasibility of autotransplantation of cryopreserved impacted third molars.

Key Words: Autotransplantation; Cryopreservation; Molar, third; Periodontal ligament

Corresponding Author: **Sun-Jong Kim**
Department of Oral and Maxillofacial Surgery, Ewha Womans University Medical Center, 1071 Anyangcheon-ro, Yangcheon-gu, Seoul 07985, Korea
TEL : +82-2-2650-2720, FAX : +82-2-2650-2754, E-mail : oralsurgeonsj@gmail.com

Received for publication August 1, 2015; Returned after revision December 20, 2015; Accepted for publication December 23, 2015

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Introduction

Autotransplantation is a commonly used surgical procedure for replacement of a missing tooth. If a donor tooth is healthy and appropriate, the prognosis of autotransplantation has been known to be excellent. Andreasen et al.[1] reported an autotransplantation success rate of ≥95%. Czochrowska et al.[2] reported a success rate of 79% and a transplant survival rate of 90% after follow-up of 17 to 41 years, and concluded that autotransplantation is an acceptable treatment modality for missing teeth. The success rate of autotransplantation depends on various factors including initial stability, the exposure time of donor teeth, the length of operating time, and the manipulation/protection of the periodontal ligament (PDL)[3]. Above all, the survival and maintenance of periodontal cell functions seem to be essential for the repair process and for the good prognosis of grafted teeth[4]. However, since autotransplantation is sometimes difficult to perform immediately after tooth extraction, the tooth may need to be stored before its usage. A tooth bank should be established for future autotransplantation[5] if the prognosis of injured teeth is doubtful[6] or there is a need to obtain sufficient space through orthodontic procedures[5].

Cryopreservation has been extensively studied as a method to store teeth for long periods. Bartlett and Reade[7] reported a successful tooth autotransplantation after cryopreservation at –196°C. Also, Schwartz and Rank[8] stated that cryopreserved human maxillary premolars healed sufficiently to have radiologically normal PDLs even 4 years after replantation. Conventional cryopreservation has subsequently been shown to maintain the membrane integrity, cell viability, and differentiation capability of the PDL[3,5,9]. However, we must evaluate various types of injuries to the cells during each step of the process, including ice injury during cooling to –196°C, chemical toxicity, and osmotic changes due to cryoprotectants. Numerous studies have been conducted to establish a cryoprofile, including rate-controlled freezing[10], application of magnetic fields[11], and vitrification[12].

To date, previous studies have focused on replantation of mature premolars after cryopreservation mainly for orthodontic purposes[6,10,13]. However, the premolar area has a limited indication for autotransplantation and replantation leads to spatial and functional imbalance between premolars and molars during rehabilitation. There have been few studies on impacted third molars. Although the composition of the PDL in premolars and the third molars is similar, there are significant differences in function due to their shape and size.

This study was conducted to determine cell viability and differentiation capability of human PDL cells and to elucidate the effects of cryopreservation on the activity of human third molar PDL cells by comparing PDL cells with and without cryopreservation.

Materials and Methods

1. Isolation and Culture

Human PDL cells were obtained from specimens after extraction of the immature impacted third molar. Before the extraction, each volunteer signed an informed consent form. A total of 29 human third molars were obtained from 12 healthy patients. The mean age of the patients was 24.3±3.2 years. All molars were fully impacted, and they were extracted by a single dentist. This study excluded patients who had (1) periodontal disease, (2) dental root disease, (3) cystic lesions, and (4) infectious diseases, including human immunodeficiency virus and hepatitis B virus. This study was approved by the Ethics Committee of the Ewha Womans University, Seoul, Korea (226-1-37). Immediately after the teeth were extracted, they were stored in F-medium[14], which was composed of Dulbecco's modified Eagle's medium (Gibco BRL; Life Technologies, Grand Island, NY, USA) and Ham's

nutrient mixture F-12 (Gibco BRL) at a ratio of 3 to 1, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/μl), streptomycin (100 μl/ml) and Fungizone (0.3 μg/ml) (Gibco BRL).

The PDL was carefully separated from the surface of the middle third part of the third molar roots by scraping it into a Petri dish with 10 ml of F-medium. Separated tissue was minced into pieces smaller than 0.5 mm^3. After washing several times in F-medium, the fragments of the PDL were placed as explants in plastic culture flasks with 20 ml of F-medium. The culture plates were maintained at 37°C in a 5% CO_2 humidified atmosphere to allow the tissue to attach to the walls. The fibroblasts were trypsinized and subcultured when growing cells covered at least 50% of the entire surface. PDL cells from the third passage (P3) were used for experiments.

2. Cryopreservation and Thawing

At confluence, tissue culture dishes (P3 cells) were washed with PBS twice and trypsinized using 1 ml of 0.25% trypsin and 0.08% ethylenediamine tetraacetic acid, and harvested in F-medium. Then, PDL cells were harvested in F-medium and centrifuged (Bench-top Centrifuge 416G; Gyrogen, Seoul, Korea) at 1,000 rpm for 3 minutes. The PDL cells were divided into 2 groups. The control group was fresh PDL cells (P3), and the experimental group was PDL cells slowly frozen with a programmed freezer. As a cryoprotectant, 10% dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) was added and maintained at 20°C for 10 minutes for equilibration[4]. The cells were placed in a programmable freezer (2100 system; Custom Biogenic System, Van Dyke, MI, USA) and slowly frozen from 4°C to –35°C at a velocity of –0.5°C/min, and were left alone at –35°C for 10 minutes. Thereafter, the cells were stored at –196°C in a liquid nitrogen tank (CHART/MVE, Marietta, GA, USA) for 7 days before thawing[3].

To evaluate the vitality of the PDL cells, vials were retrieved from storage, immediately thawed in a 37°C water bath for 3 minutes, and maintained at room temperature for 5 minutes. After the cells were equilibrated in F-medium and 10% FBS, they were centrifuged at 1,000 rpm for 3 minutes. The supernatant was discarded and resuspended in F-medium.

3. Evaluation of Cell Viability, Growth Capacity, and Morphological Assessment

For the evaluation of cell viability (membrane integrity), the trypan blue exclusion method was used. The cell suspension was mixed with 0.4% trypan blue (Gibco BRL). The numbers of non-colored surviving cells and blue-colored apoptotic cells were counted using a hematocytometer. Cell viability was compared in terms of the ratio of surviving cells to apoptotic cells.

For the comparison of growth capacity between 2 groups, MTT assay (3,4[4,5-dimethylthiazol-2-y1]-2,5=diphenyltetrazolium bromide) was carried out[15]. Each experiment was repeated at least 3 times. Cell suspensions from each group were placed in a 96-well tissue culture plate (Microtest™ 96; BD Falcon Co., Jersey City, NJ, USA) at a concentration of 2×10^4 cells/well at 37°C overnight. Then, 150 ml of 0.05 mg/ml MTT solution (Sigma Chemical Co.) was poured into each well and incubated for 3 hours at 37°C. The formazan crystals were dissolved by the addition of 100 ml of Me_2SO (Sigma Chemical Co.) to each well and absorbance was measured at 570 nm using an ELISA microplate reader (Bench Mark Plate Reader; Bio-Rad, Hercules, CA, USA). The same experiment was conducted on days 3, 5, 7, 10, and 12.

A set of stock cell vials was used for morphological assessment. One week after storage, the cryovials were thawed as described for the viability assay. Then, 1×10^4 cells/ml were seeded in 35-mm^2 dishes in F-medium and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. Histological morphology of thawed cells and control fresh cells was assessed using an inverted microscope

(CKX41/inverted microscope; Olympus, Tokyo, Japan) at a magnification of 40×.

4. Statistical Analysis

Initially, the Mann-Whitney U-test was used to compare trypan blue results between the cryopreserved and control groups. The independent t-test was used to compare growth capacity between 2 groups at each time point (days 1, 3, 5, 7, 10, and 12).

To investigate growth trends over time, we used a linear mixed model (LMM) analysis of repeated measures with MTT assay values as a continuous outcome variable. Restricted maximum likelihood estimation and Type 3 tests of fixed effects were used. P-values of <0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Co., Armonk, NY, USA).

Result

1. Cell Viability (Membrane Integrity) and Growth Capacity

The results of both cell viability and growth capacity assays are shown in Table 1. Cell viability (membrane integrity) measured by the trypan blue exclusion test was not significantly different between 2 groups (P>0.05, Mann-Whitney test). Growth capacity measured by MTT was not significantly different between 2 groups at any time point (P>0.05, independent t-test).

2. Trend of Growth over Time

The mixed model analysis of repeated measurements of growth over time revealed no significant difference between 2 groups (β=–0.0009; 95% confidence interval [CI], –0.0021~0.0003; P=0.138). The regression coefficient (β) of the trend of growth over time in both groups was 0.089, with statistical significance (95% CI, 0.082~0.095; P<0.001).

Table 1. Comparison of cell viability and growth capacity between cryopreserved and fresh cells

	Cryopreserved group	Control group	P-value*
Trypan blue (%)	87.9±3.78	84.4±5.83	0.128
MTT assay (OD)			
Day 1	0.056±0.004	0.054±0.004	0.153
Day 3	0.081±0.005	0.082±0.008	0.335
Day 5	0.106±0.011	0.103±0.007	0.059
Day 7	0.131±0.022	0.133±0.010	0.479
Day 10	0.135±0.026	0.148±0.047	0.077
Day 12	0.159±0.030	0.164±0.054	0.591
Day 14	0.157±0.029	0.177±0.070	0.153

MTT: 3,4[4,5-dimethylthiazol-2-y1]-2,5=diphenyltetrazolium bromide, OD: optical density.

Values are presented as mean±standard deviation.

There was no statistical difference between 2 groups over time as assessed by linear mixed model (LMM) analysis of repeated measures using MTT assay values (no group×time interaction, P=0.138).

Time trend of growth in both groups; estimated coefficient (β): 0.089 (0.082~0.095), P<0.001.

*Acquired by the Mann-Whitney U-test for trypan blue, and the independent t-test for MTT assay.

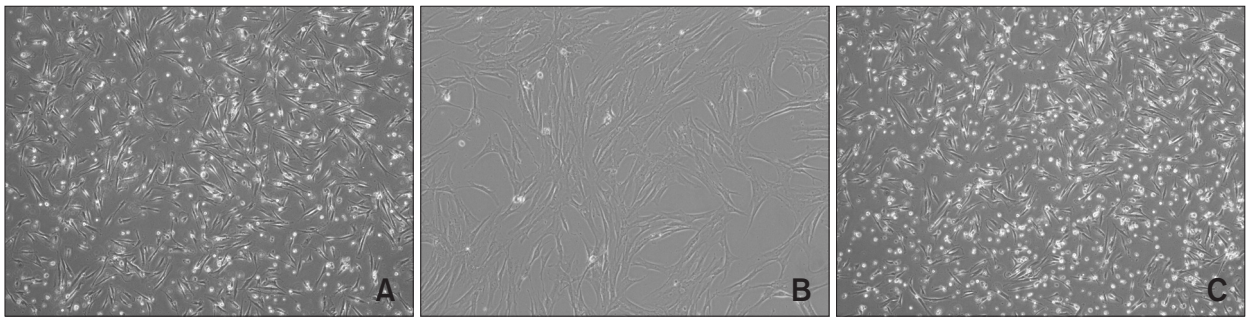

Fig. 1. Inverted microscopic images for the evaluation of cell morphology. The cultured cells show a spindle-shaped fibroblast-like morphology in both the cryopreserved group (A: ×40; B: ×100) and control group (C).

3. Morphological Evaluation

Images taken with an inverted microscope were used to observe changes in the morphology of cultured cells after cryopreservation. Cells from both fresh and cryopreserved teeth were fibroblast-like in appearance, and there were no significant differences in morphology between 2 groups (Fig. 1).

Discussion

As mentioned above, most studies on transplantation of cryopreserved teeth have focused on premolars, which have limited surgical indications for clinical disorders. The regeneration process of cryopreserved teeth after transplantation is complex, so it is difficult to determine which factors and cell types are associated with the regeneration process. Our study primarily focused on one of the most important factors, the survival and functional status of PDL cells[16,17]. We compared the cellular activity of slowly-frozen PDL cells to that of never-frozen PDL cells and evaluated the effects of cryopreservation on human third molars.

To minimize cell damage due to freezing in our experiments, slow programmable freezing was used. Andreasen and Hċkansson[16] proposed that teeth should be slowly frozen to –35°C at a velocity of –0.5°C/min, to –190°C at a velocity of –6°C/min, and then quickly to –196°C[16]. However, our slow programmable freezing protocol was to cool at –0.5°C/min from 4°C to –35°C and hold there for 10 minutes, then plunge the samples into liquid nitrogen at –196°C and store for 1 week before thawing[3]. Kawasaki et al.[10] reported that slow programmable freezing reduces damage to viable tissue. Attempts have been made to increase the viability of PDL cells during freezing: (1) cryopreservation of PDL cells with magnetic fields[11,18] and (2) programmed cryopreservation under pressure[19]. To prevent cellular damage due to ice formation during thawing[20], cryovials in LN_2 were directly plunged into a 37°C water bath for 3 minutes and then kept at room temperature for 5 minutes[21]. Slow equilibration was achieved by serial dilution using medium consisting of 90% F-medium and 10% FBS. Although DMSO has been widely used as a cryoprotectant to inhibit intracellular ice formation during the cryopreservation process, its chemical toxicities and osmotic effects have created serious problems[22]. Schwartz et al.[4] demonstrated that 10% DMSO is a suitable cryopreservation medium and protects PDL cells better during the cryopreservation process. Their freezing protocol was stepwise equilibration at room temperature to a final DMSO concentration of 10% followed by immersion in nitrogen solution. Similarly, we used stepwise equilibration at 20°C to a final concentration of 10% DMSO.

The percentage of cells which excluded trypan blue in the cryopreserved group was not significantly different than that in the control group. However, it is worth noting that membrane integrity testing reveals the status of cell membranes at a certain time, but does not offer insights into linear behavior and the potential longitudinal properties of these cells[23]. In order to study the long-term effects of cryopreservation on PDL cells, it is more appropriate to assess the growth capacity of these cells[5].

We used the MTT assay to evaluate the growth capacity of PDL cells. This is one of the widely used methods to assess the survival of cultured cells and tissues and has been employed to measure cellular activity after cryopreservation[3,6]. In our study, absorbance was not significantly different between the cryopreserved and control cells as assessed by MTT. In addition, inverted microscopy revealed that cell morphology was not significantly different between the cryopreserved and control cells and that both cryopreserved and control cells represented viable PDL cells with a normal spindle shape. These results are in good agreement with those of previous studies which reported that cryopreservation does not affect the biological

properties of PDL cells[24-27].

Advantages of autotransplantation over other rehabilitation methods include the preservation of proprioception, acceptable healing of periodontal tissues, and its promotion of alveolar regeneration[6,9]. Although third molars have been successfully autotransplanted in clinical practice[2,28,29], there is still no definitive evidence for the cryopreservation of impacted third molars. Surgical trauma should be avoided while extracting impacted third molars. Autotransplantation should be considered in patients who need odontectomy and whose dental root is formed by more than 75%[16], those who are 24 years or older, and in those whose bone mineralization is highly progressed. During tooth extraction, bone cutting should be minimal around the crown area. Teeth with severe mesioangulation and distoangulation impactions are vulnerable to periodontal injury due to luxation. Although PDL mesenchymal cells are more resistant to injury than pulpal mesenchymal cells[26], there is no direct evidence for this phenomenon in impacted third molars. Thus, a completely intact PDL is required for successful tooth preservation and transplantation.

Conclusion

This study was conducted to compare cell viability and growth capacity between cryopreserved and fresh impacted third molar PDL cells. Cell viability and growth capacity were not significantly different between 2 groups. This study demonstrates that cryopreservation does not affect the biological properties of PDL cells, supporting the feasibility of autotransplantation of cryopreserved impacted third molars.

Conflict of Interest

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

Acknowledgement

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0013353) & Ewha Womans University Research Grant of 2010.

This article was based on the PhD dissertation of TY Kim from Ewha Womans University, Graduate School of Clinical Dentistry.

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