Evaluation of vitrification for cryopreservation of teeth

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Purpose: The aim of this study was to investigate whether vitrification in the cryopreservation of periodontal ligament (PDL) cells could be useful for tooth banking.

Methods: In step 1, primary cultured human PDL cells were cryopreserved in 100% conventional cryopreservation media and 100% vitrification media (ESF40 media) in different temperatures for 2 weeks. In step 2, a series of modified vitrification formulae named T1 (75% vitrification media + 25% F-media), T2 (50% vitrification media + 50% F-media) and T3 (25% vitrification media + 75% F-media) were used to store PDL cells for 2 weeks and 4 weeks in liquid nitrogen. MTT assay was performed to examine the viability of PDL cells.

Results: Maximum cell viability was achieved in cells stored in 100% conventional cryopreservation media at -196°C (positive control group) in step 1. Compared to the positive control group, viability of the cells stored in 100% vitrification media was very low as 10% in all test conditions. In step 2, as the percentage of vitrification media decreased, the cell viability increased in cells stored for 2 weeks. In 4-week storage of cells in step 2, higher cell viability was observed in the T2 group than the other vitrification formulae while the positive control group had the highest viability. There was no statistically significant difference in the cell viability of 2-week and 4-week stored cells in the T2 group.

Conclusions: These observations indicate 100% vitrification media is not successful in PDL cell cryopreservation. Conventional cryopreservation media is currently the most appropriate media type for this purpose while T2 media would be interesting to test for long-term storage of PDL cells.

Keywords: Cryopreservation, Periodontal ligament, Tissue banks.

INTRODUCTION

Preservation of teeth for future use, mainly for autografts and for selected allografts, shows potential for organization of a tooth bank. The proper storage of donor teeth in order to maintain the viability and differentiation capability of periodontal ligament (PDL) cells is an important factor in determining success after autotransplantation. Transplantation of a healthy tooth has been reported to induce the regeneration of the destroyed alveolar bone through the differentiation capability of PDL cells [1].

Cryopreservation is the method of choice for long term storage of living tissues. Despite its disadvantages, this technique has been practiced for many decades in preserving the vital functions of the cells of many types of mammalian and human tissues. A method for cryopreservation of mature teeth has been developed by modifying the techniques used for cryopreservation of mammalian embryos [2]. Successful
autotransplantation and allotransplantation of cryopreserved teeth using this technique have been reported [2,3]. The conventional cryopreservation method has been proven to maintain the membrane integrity, viability, and differentiation capability of PDL cells [3-5]. Although the functions of PDL cells are saved by the conventional cryopreservation method, it can cause various types of injuries to the cells during each step of the process. The primary injury is caused by the formation of intracellular ice (ice crystallization) during cooling to -196°C. Secondly, the cryoprotectants that are added to prevent primary injury could cause chemical toxicity and osmotic change, causing swelling of the cells [6].

Tooth cryopreservation should preserve both tooth hard tissue and soft tissues for a successful replantation sparing the masticatory functions. A previous study by Oh et al. [3] reported the occurrence of a longitudinal fracture in 25% of conventionally cryopreserved teeth during a hardness test. Therefore, this study was designed to develop a new freezing method for tooth preservation.

Vitrification, which is defined as the solidification of a solution by an extreme elevation in the viscosity without crystallization, has been developed as an alternative cryopreservation procedure aiming to minimize tissue injuries [6]. A variety of cells, and more recently tissues, have been successfully cryopreserved using vitrification [7-10]. This approach has been applied to improve functional survival of living tissues after freezing and thawing.

Although vitrification has been successfully applied to many cells and tissues such as embryos, monocytes, endothelial cells, vascular grafts, and skin grafts, no attempt has been taken so far to vitrify teeth, tooth buds or PDL cells to the best of our knowledge.

As vitrification has shown cell functional and survival recovery superior to conventional cryopreservation methods, in this study we examined the effects of vitrification on PDL cells. For successful tooth transplantation, the integrity of PDL cells is an utmost need; therefore, the purpose of this study was to evaluate whether vitrification can be useful for tooth cryopreservation. In this study we tested and compared the viability of PDL cells after vitrification or conventional cryopreservation, each for two and four weeks.

**MATERIALS AND METHODS**

**Cell isolation and culture**

Human PDL tissue was obtained from patients having healthy first premolar teeth extracted for orthodontic purposes. Prior to extraction, informed consent was obtained. The study was approved by the ethics committee of the Yonsei University College of Dentistry.

Each extracted tooth was gently washed with saline and immediately placed in F- medium, which was composed of Dulbecco’s modified Eagle’s medium (Gibco BRL, Life Technologies, Grand Island, USA) and Ham’s nutrient mixture F-12 (Gibco BRL, Life Technologies, Grand Island, USA) in a ratio of 3 to 1, supplemented with 10% fetal bovine serum and antibiotics, consisting of penicillin (100 units/mL), streptomycin (100 μL/mL), and Fungizone (0.3 μg/mL) (Gibco BRL, Life Technologies, Grand Island, USA).

PDL was gently separated from the surface of the root by scraping it into a Petri dish with 10 mL F-media. The PDL attached to the middle third of the root was cut from the root surface using a scalpel knife to avoid incorporation of gingival cells. Separated tissue was minced in to tiny pieces less than 0.5 mm3. After washing several times in phosphate buffered saline (PBS), the fragments of the PDL were placed as explants in plastic culture flasks with 20 mL of F-media. The culture flasks were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide to allow the tissue to attach to the walls. The culture medium was not changed until outgrowth of cells was seen. Culture media were then changed every 2nd or 3rd day. At confluence, the fibroblasts were trypsinized and subcultured. PDL cells from passage 6 to passage 8 were used for the experiment to achieve their maximum proliferative potential and homogeneity.

**Preparation of cryopreservation media**

The conventional cryopreservation media was prepared by mixing 80% F-medium with 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) (Me₂SO; Sigma Chemical, St. Louis, USA). Vitrification media was prepared according to the formula developed by Kasai and Mukaida [6] named ESF40. This comprises 40% ethylene glycol (EG) (WAKO Pure Chemical Industries Ltd., Osaka, Japan), 18% Ficoll 70 (SIGMA, St. Louis, USA), and 0.3 M sucrose (Junsei Chemical Co. Ltd., Tokyo, Japan) dissolved in F-medium.

**Cryopreservation procedure**

**Step 1**

As a starting point, 100% vitrification media and 100% conventional media were each used as storage media. The cell lines were grouped by storage conditions as indicated in Table 1. At confluence, tissue culture dishes were washed with PBS 3 times and trypsinized using 1 mL of 0.25% trypsin and 0.08% ethylenediamine tetracetic acid (EDTA), and harvested in F-media. Five cryotubes were prepared for each experimental group. One mL of resuspended cells was aliquoted into each cryotube, and immediately placed on ice after proper tightening of the caps. For the V3 group, the cryopro-