Evaluation of mechanical and histological properties of cryopreserved human premolars under short-term preservation: A preliminary study

Shu-Li Lin a, Sheng-Yang Lee b,c,d, Yen-Chuang Lin b, Yen-Hua Huang e, Jen-Chang Yang c,f, Haw-Ming Huang g*

a Dental Department, Cathay General Hospital, Taipei, Taiwan
b School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan
c Center for Teeth Bank and Dental Stem Cell Technology, Taipei Medical University, Taipei, Taiwan
d Orthodontic Department of Wan-Fang Hospital, Taipei, Taiwan
e Department of Biochemistry, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
f School of Dental Technology, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan
g Graduate Institute of Biomedical Materials and Tissue Engineering, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan

Received 9 April 2012; Final revision received 4 June 2012
Available online 2 June 2013

KEYWORDS
cryopreservation; program freezer; tooth

Abstract  Background/purpose: The aim of this study was to investigate whether storage of extracted teeth in transportation solution for 24 hours has a negative effect on the cell density and mechanical properties of thawed pulp tissue extracted from frozen intact teeth.
Material and methods: Human premolars were kept in transportation solution for 0 hours or 24 hours. For each transportation time, the tested teeth were divided into two experimental groups: teeth that had been frozen in a magnetic programmed freezer (PF, n = 5) and in a traditional freezer at −20°C (TF, n = 5). The tested teeth were then stored at −150°C for 7 days. After thawing, the extracted pulp was subjected to a histological examination and mechanical testing.
Results: Storage in transportation solution for 24 hours had no significant negative effects on the histological or mechanical properties of the pulp tissue extracted from the cryopreserved intact teeth. The elastic modulus of pulp from the teeth that had been frozen at −20°C was significantly
Introduction

The effect of cryopreservation on various tissues and cells is an important issue for regenerative medicine. Recently, cryopreservation of teeth for autotransplantation and isolation of dental pulp stem cells (DPSCs) was reported to be feasible by several scholars. Almost all of those studies focused on the effects of the cryopreservative process on the viability of periodontal tissues, which is the most important factor for successful transplantation. Recently, DPSCs in permanent teeth have gradually become the focus of tissue engineering. However, isolation of DPSCs is laborious and time-consuming. Because previous studies showed that viable DPSCs can be recovered from cryopreserved intact teeth, cryopreservation of whole teeth or isolated tissue might be more effective than extracting DPSCs immediately after tooth extraction.

In clinical practice, extracted teeth should be stored in a transportation solution before being sent to a laboratory for banking. Woods et al showed that various factors, including time after tooth extraction, the concentration of the cryoprotective agent, the concentration of frozen cells, and the storage temperature, affected the recovery rate of pulp cells. In 2008, Perry et al investigated how soon after extraction DPSCs could successfully be isolated. In their experiment, extracted teeth were immersed in various transportation solutions and stored at 4°C for 0–120 hours before the DPSCs were isolated. Their results indicated that the time after tooth extraction was positively associated with the health of preserved pulp cells.

Extracellular components of the pulp include various types of soft tissue, such as collagen fibers and ground substance. For years, it was believed that these soft tissues in the pulp could not be cryoprotected. Recently, however, Laureys et al showed that teeth could revascularize after being cryopreserved in a tooth bank for 1 week. Interestingly, a previous animal study demonstrated that the storage time between tooth extraction and the freezing procedure is an important factor influencing the cryopreservation of pulp cells. However, whether storage of extracted teeth in transportation fluid prior to the freezing process influences the viability of thawed human pulp tissue is still unknown. Thus, the aim of this study was to mechanically and histologically investigate the influence of the transportation period before cryopreservation on human pulp tissue in cryopreserved intact teeth.

Materials and methods

Cryopreservation of intact teeth

In this experiment, 40 normal premolars from 20 patients were collected from adults ranging in age from 18 years to 30 years at the Department of Orthodontics, Wan-Fang Medical Center, Taipei Medical University, Taipei, Taiwan. All experimental protocols stated in this study were approved by the Committee on Human Research, Taipei Medical University. This information was also provided to the patients whose teeth were collected, and an agreement was signed by all patients before the experiment. Immediately after extraction, a tooth was cleaned with phosphate-buffered saline (PBS) and stored in a cryoprotectant (BAMBANKER; Lymphotec, Tokyo, Japan). The teeth were divided into three groups (Fig. 1). Teeth in the programmed-freezing group (PF group) were cryopreserved in a magnetic programmed freezer (Cell Alive System; ABI, Chiba, Japan). Briefly, the teeth were transported at 4°C and then placed in the programmed freezer at −5°C. The teeth were maintained at that temperature for 15 minutes, and then cooled at a rate of −0.5°C/min until the temperature reached −32°C. Teeth in the traditional freezing group (TF group) were extracted, immersed in commercialized dimethyl sulfoxide (DMSO)-free cryoprotectant (modified-BAMBANKER; Lymphotec, Tokyo, Japan), and then frozen in a traditional freezer at −20°C. After the freezing procedure, all experimental teeth were transferred to a −150°C freezer (MDF-11561; Sanyo, Osaka, Japan) and stored for 7 days. The extracted premolars in the two experimental groups were kept in transportation solution for 0 hours or 24 hours, with five samples used for each of the following experimental conditions. The control group comprised premolars that had been extracted from the opposite side of the same patient in each experimental group. The control teeth were not subjected to the freezing procedure or 7-day cryopreservation.

Histological examination of pulp tissue

After thawing at room temperature, teeth were fixed in 10% formalin and a decalcification solution in 10% formic acid.

![Flow chart and sample sizes of the entire experiment.](image)

**Figure 1** Flow chart and sample sizes of the entire experiment. PF = programmed freezing group; TF = traditional freezing group.
for 48 hours as described elsewhere. Tooth samples were then horizontally cut into cubes and dehydrated using a graded series of alcohol. After dehydration, the tooth cubes were embedded in paraffin and sliced into 6-μm sections with a sliding microtome (Leica LM2500; Meyer Instruments, Langham Creek, Houston, TX, USA). Slices were then dewaxed, stained with hematoxylin and eosin (H&E), and examined under an optical microscope.

Eight microscopic images were randomly taken of each pulp slice. For each microscopic image, two 40 × 15-μm squares were respectively defined in the odontoblast region and the cell-rich zone (Fig. 2). In this experiment, a cell number index (CNI) was defined as the ratio between cell numbers counted in the frozen premolar and those in the non-frozen tooth extracted from the opposite side of the same patient. CNI was used to represent the cell density.

In this experiment, a quantitative analysis was performed by comparing cell densities obtained under the various experimental conditions. An analysis of variance (ANOVA) with Tukey’s post-hoc test was used for statistical analysis.

Mechanical tests of pulp tissue

To test the elastic modulus of the test pulp, thawed pulp tissues were fixed to an elastic modulus-testing device (EX-9928; PASCO, Roseville, CA, USA). A static pulling force was then applied to the test samples at a rate of 0.5 mm/s until the samples fractured. For the two experimental groups and the control group, five and 10 specimens were tested, respectively. For each specimen, the stress and strain in the elastic region were recorded and used to calculate the elastic modulus. The mean modulus value of each experimental group was recorded.

Results

To examine the effect of transportation time on dental pulp cells, thawed samples were fixed, sliced, and stained with H&E. Cell counts of pulp slices from the odontoblastic region and the cell-rich zone are given in Table 1. Among the samples that had not been stored in transportation solution, the mean CNI value in the odontoblast region was significantly greater in the PF group (0.97 ± 0.01) than in the TF group (0.59 ± 0.3) (P < 0.05) (Fig. 3A). CNI values in the cell-rich zone were 0.88 ± 0.03 in the PF group and 0.67 ± 0.3 in the TF group (Fig. 3B). There was no significant difference between these two groups. For samples stored in transportation solution for 24 hours before undergoing the freezing procedure, mean CNI values in the odontoblast region (Fig. 3A) and the cell-rich zone (Fig. 3B) were significantly lower in the TF group than in the PF group (P < 0.05). There were no significant differences in mean CNI values in the odontoblast and cell rich zones between PF samples that had been pretreated with transportation solution and PF samples that were untreated.

As seen in Fig. 4, pulp extracted from samples that had not been exposed to transportation solution before undergoing the freezing procedure had an elastic modulus in the range 2.2–2.7 MPa. There was no significant difference in the elastic modulus of extracted pulp between samples that had been exposed to transportation solution and samples in the control group. However, the elastic modulus increased by fivefold in samples that had been exposed to transportation fluid for 24 hours followed by storage at −20°C (P < 0.05).
Discussion

For years, it was thought that pulp tissue would not survive during the banking period. Recently, however, Lee et al showed that mesenchymal stem cells in pulp tissue have the ability to recover after the cryopreservation process. In this study, we found that storage in transportation solution for 24 hours did not affect the histological or mechanical properties of pulp tissues after thawing from short-term cryostorage (Figs. 3 and 4). Because the percentage of dental pulp stem cells in the pulp cell population is about 10%, the result presented in this study is consistent with that reported by Brandon et al who showed that DPSC cultures can be established from about 80% of extracted human third molars within 24 hours of extraction if teeth are stored at 4°C in transportation solution. Because immunostaining of proliferation markers was not performed in this study, the real viability of pulp cells and/or DPSCs with different cryopreservation processes could not be determined. This is a limitation of the current study.

Pulp tissue and cells can be cryopreserved using three pre-processing samples: digested pulp tissue, intact pulp tissue, and whole teeth. Wood et al compared the efficiency of DPSC cryorecovery among the three sample types and found that optimal results were obtained after cryopreservation of isolated pulp tissue, followed by digestion and culture post-thaw. Both liquid nitrogen and a programmed freezer were used for cryopreserving intact teeth and isolating DPSCs post-thawing. This is because these methods can provide a faster decrease in temperature. Because the temperature of the storage chamber can pass through the ice-crystal formation temperature faster, ice crystals produced by these two methods can be reduced. In a cryostorage experiment of intact molars, Temmerman et al showed that there was a positive correlation between the dimensions of the apical foramen and pulpal viability post-thawing. This is because the dimensions of the apical foramen of the tooth should be large enough to enable the cryoprotective agent to penetrate and provide protection for pulpal tissues. In this study, the dimensions of the apical foramen were not taken into account as a parameter. However, our results confirmed that short-term cryopreservation of pulp cells in intact teeth is possible.

The typical elastic modulus for pulp tissue is 2.07 MPa. As seen in Fig. 4, we confirmed that the elastic modulus of pulp tissue is about 2 MPa. The mechanical properties of the programmed-frozen/thawed tissue did not markedly differ from those of tissue in fresh culture. However, we found that the elastic modulus of pulp from teeth that had been stored in transportation solution for 24 hours followed by exposure to -20°C increased by almost 2.5-fold.

| Table 1 | Cell counts of pulp slices.  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PF</td>
</tr>
<tr>
<td>Cell rich zone (10⁵ cells/mm²)</td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>24 h</td>
<td>6.6 ± 1.6</td>
</tr>
<tr>
<td>Odontoblastic region (10⁵ cells/mm²)</td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>24 h</td>
<td>19.8 ± 8.6</td>
</tr>
</tbody>
</table>

PF = programmed freezing; TF = traditional freezing.
Diaz-Tenorid et al compared different freezing and thawing treatments on muscle properties and found that the freezing process promoted stiffness and hardness. This is because there was marked water loss in the frozen sample after thawing. They concluded that the freezing method rather than the thawing method affected the final thawed sample’s physical properties. This finding might explain the results shown in Fig. 4.

In conclusion, the results of this study showed that storage in transportation solution for 24 hours followed by exposure to programmed freezing did not negatively affect the cell density or elastic modulus of the pulp tissues.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This study was supported by a grant from Cathay General Hospital (99CGH-TMU-09) and partially from Li-Jian Biotechnology (99TMU-ICC-003). The authors would like to thank ABI (Chiba, Japan), Three Brackets (Hiroshima University, Japan), and the Center for Teeth Bank and Dental Stem Cell Technology (Taipei Medical University) for freezing instrument support.

References