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OPTIMIZED CRYOPRESERVATION METHOD FOR HUMAN DENTAL PULP-DERIVED STEM CELLS AND THEIR TISSUES OF ORIGIN FOR BANKING AND CLINICAL USE

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Abstract

Dental pulp is a promising source of mesenchymal stem cells with the potential for cell-mediated therapies and tissue engineering applications. We recently reported that isolation of dental pulp-derived stem cells (DPSC) is feasible for at least 120 hours after tooth extraction, and that cryopreservation of early-passage cultured DPSC leads to high-efficiency recovery post thaw. This study investigated additional processing and cryobiological characteristics of DPSC, ending with development of procedures for banking. First, we aimed to optimize cryopreservation of established DPSC cultures, with regards to optimizing the cryoprotective agent (CPA), the CPA concentration, the concentration of cells frozen, and storage temperatures. Secondly, we focused on determining cryopreservation characteristics of enzymatically digested tissue as a cell suspension. Lastly, we evaluated the growth, surface markers and differentiation properties of DPSC obtained from intact teeth and undigested, whole dental tissue frozen and thawed using the optimized procedures. In these experiments it was determined that Me₂SO at a concentration between 1 and 1.5M was the ideal cryopreservative of the three studied. It was also determined that DPSC viability after cryopreservation is not limited by the concentration of cells frozen, at least up to 2×10^6 cells/mL. It was further established that DPSC can be stored at -85°C or -196°C for at least six months without loss of functionality. The optimal results with the least manipulation were achieved by isolating and cryopreserving the tooth pulp tissues, with digestion and culture performed post-thaw. A recovery of cells from >85% of the tissues frozen was achieved and cells isolated post thaw from tissue processed and frozen with a serum free, defined cryopreservation medium maintained morphological and developmental competence and demonstrated MSC-hallmark trilineage differentiation under the appropriate culture conditions.

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Keywords

Mesenchymal stem cells; dental pulp stem cells; adult stem cells; cryopreservation; tissue engineering; stem cell banking

INTRODUCTION

The term “stem cell” generally refers to a cell possessing the ability to self-replicate and give rise to daughter cells which undergo an irreversible, terminal differentiation process [2,10]. Stem cells from post-natal human origin have been studied extensively from sources such as the epidermis, gastrointestinal epithelium, adipose tissue, umbilical cord blood and bone marrow. To date, the hematopoietic stem cells originating in the bone marrow have arguably been the most extensively studied [13]. Also first identified in the bone marrow is a population of multipotent mesenchymal stromal cells (“mesenchymal stem cells” or MSC) which contribute to the formation of multiple mesodermal tissue types, such as bone, muscle, cartilage, ligaments, tendons, and adipose tissue [4]. MSC or MSC-like cells have now been isolated from various tissues and are of interest to many researchers and clinicians due to their easy isolation, ability to greatly expand in culture and many potential uses in cell-mediated therapies and tissue engineering applications [8].

One source from which MSC have not been as thoroughly studied is dental tissue. Recently, several groups have initiated investigation of this potential source and have begun to examine the properties of stem cells isolated from dental pulp, periodontal ligament and periapical follicles of adult teeth, as well as from deciduous teeth [8,9,12,16,19]. As these cells are characterized and their importance realized, reproducible methods for harvest, banking and distribution become critical.

Results of recently published studies on cryopreservation of dental-tissue derived mesenchymal stem cells have been promising, but are preliminary at best. To date the most successful cryorecovery procedures have used previously-established cell cultures [14,21]. However, while these studies demonstrated good recovery post-thaw, the cells were derived from relatively low numbers of teeth, and this method requires extensive up-front processing. One study evaluated cryopreservation of intact periodontal ligaments with processing and culture establishment post-thaw; however this group reported a great reduction in colony development as compared to controls [19].

Our group recently reported the first study in which the processing of dental pulp-derived MSC (DPSC) from harvest to storage was considered using large numbers of teeth [18]. In that study we reported that isolation of DPSC is feasible for at least 120 hours after tooth extraction, and that cryopreservation of established early-passage cultured DPSC leads to high-efficiency recovery after thawing. Furthermore, we demonstrated recovery of viable DPSC after cryopreservation of intact teeth, suggesting that minimal processing may be needed for the banking of samples with no immediate plans for expansion and use.

To optimize methods for banking DPSC, the optimum cryopreservation process should be straight forward and effective when applied to the tissue as a whole, with the idea that stem cells would be extracted post-thaw. The rationale for this is to preserve clinical samples for subsequent stem cell recovery, since immediate cryopreservation of tissues will be more practical than direct primary isolation of stem cells, which requires additional equipment and personnel [7]. Indeed, isolating DPSC can be laborious, time-consuming and expensive, especially while employing current good tissue practice (cGTP) standards for clinical use of the cells; therefore, cryopreservation of whole teeth or isolated tooth tissues may be

advantageous for the banking of specimens from which DPSC cultures are not immediately needed. If cells are immediately needed from tissues, cryopreservation procedures need to be as efficient as possible to maximize the utility of the material.

In the present study, we now report results of optimized methods for cryopreservation and processing of DPSC and their respective tissues of origin for banking and to allow further study of the potential therapeutic uses of these cells. To that end, we further investigated additional processing and cryobiological characteristics of DPSC. First, we aimed to optimize cryopreservation of established DPSC cultures, with regards to optimizing the cryoprotective agent (CPA), the CPA concentration, the concentration of cells frozen, and storage temperatures. Secondly, we focused on determining cryopreservation characteristics of enzymatically digested tissue as a cell suspension. Lastly, we evaluated the growth, surface markers and differentiation properties of DPSC obtained from intact teeth and undigested, whole dental tissue frozen and thawed using the optimized procedures.

MATERIALS AND METHODS

TISSUE PROCESSING AND CELL CULTURE

Collection and Transport of Extracted Teeth—Extracted human third molars were obtained with informed consent through a local oral/maxillofacial surgical center as discarded medical waste from patients aged 18–30. Once extracted, teeth were placed into chilled solution vessels containing 20 mL of sterile phosphate buffered saline (PBS, all reagents from Sigma Chemical, St. Louis, MO, unless otherwise noted) and transported to the lab for processing within 24 hours.

Processing and Tissue Recovery—Before digestion of dental tissue, teeth were disinfected by a protocol adopted and modified from cornea banking [1]. Teeth received several brief washes in sterile saline, followed by exposure to 1% Polyvinylpyrrolidone-Iodine (PVP-I) for 2 minutes, exposure to 0.1% sodium thiosulfate in PBS for 1 minute, and another wash in sterile PBS. This was followed by immersion in Listerine® antiseptic (Johnson and Johnson Healthcare Products, Langhorne, PA) for 1 minute, followed by several final washes in sterile PBS. The roots of cleaned teeth were separated from the crown; the ends were then clipped off and retained while the roots were split to reveal the dental pulp, which was then recovered with a curette for further processing or cryopreservation.

Tissue Digestion—Recovered dental tissue was placed into an enzymatic bath consisting of a blend of saline with Type I and Type II Collagenase with thermolysin added as a neutral protease (Vitacyte, Indianapolis, IN). Tissue was incubated in this bath at 37°C for 15–30 minutes for digestion to liberate the cells. Once digestion was complete, the enzyme mixture was neutralized by addition of medium and the cells plated in Mesencult® Complete Medium (e.g., basal medium containing MSC supplements; StemCell Technologies, Vancouver Canada) as previously described [18].

Culture and Expansion of Fresh Tissue Digest—For culture of fresh digested tissue, cells were plated immediately in a T-25 flask at a density of 1 tooth digest per flask and placed into a high humidity 37°C 5% CO₂ incubator. No selection of stem cells was performed directly, and instead selective culture was used to maximize stem cell recovery. Culture flasks were monitored daily and any contaminated flasks removed immediately and recorded. Non-contaminated flasks were monitored for cell growth, with culture medium changes taking place three times per week. After 14 days of growth, adherent cells were dissociated from flasks using 0.25% trypsin containing 1 mM EDTA and a cell count of total cells per flask obtained via standard hemacytometer counting methods.

Cells were then sub-cultured for approximately 4 weeks, with medium changes taking place three times per week with passages when cells were approximately 70% confluent in the T-flask. Following trypsin dissociation, passage 3 cells were resuspended in complete medium and were confirmed to meet minimal criteria to be called MSC by flow cytometry based on the International Society for Cell Therapy (ISCT) position paper [6]. Antibodies to human CD73, CD90, CD105, CD34, CD45, CD11b, CD19 and HLA-DR were obtained from BD Biosciences (San Jose, CA). Selected DPSC cultures were stained with the above antibodies and appropriate isotype controls per the manufacturer's instructions, and were analyzed using a FACSCalibur instrument and CellQuest software (BD Biosciences). Cells $\geq 95\%$ positive for CD73, 90 and 105, and $\leq 5\%$ positive for CD34, 45, 11b, 19 and HLA-DR were confirmed DPSC. These expanded cultures were used for further cryopreservation experiments as described below.

Cell Differentiation Assays—Cells at passage 3–4 were seeded in 6 cm dishes and cultured for 3–4 weeks in the appropriate differentiation medium. Mesencult medium containing osteogenic or adipogenic differentiation supplements (both from StemCell Technologies) were used according to the manufacturer's instructions. For chondrogenic differentiation, DPSC were cultured in chondrogenic differentiation medium consisting of Mesencult complete medium with 10^{-8} mol/l dexamethasone, 5 mg/ml ascorbic acid 2-phosphate, 10 mmol/l beta-glycerophosphate and 10 ng/ml TGF β 3 (PeproTech, Rocky Hill, NJ). Differentiated cultures were stained as previously described [18].

Flow cytometric and multilineage differentiation analyses were performed in the same manner for both fresh and frozen-thawed DPSC.

CRYOPRESERVATION EXPERIMENTS

Expanded DPSC: Optimized CPA and CPA Concentration—Cryopreservation of passage 3 cell lines from DPSC cultures derived from 4 separate tooth donors was conducted using final concentrations of 0.5M, 1.0M, and 1.5M ethylene glycol (EG), propylene glycol (1,2 propane diol; PG), and dimethyl sulfoxide (Me₂SO), respectively, prepared weight/volume in Mesencult complete medium. Cells were equilibrated to these final concentrations using a two-step process to avoid potential osmotic damage [20]. Following equilibration, cells were packaged in 2 mL cryovials containing 1 million cells per vial, and cooled at approximately $-1^{\circ}\text{C}/\text{minute}$ using a dump-freeze method consisting of suspension of vials in an isopropanol bath within a -85°C mechanical freezer (VIP Series Ultra-Low Temperature Freezer, Sanyo Scientific, Bensenville, IL) for 24 hours, followed by plunge into liquid nitrogen (LN₂) for storage at -196°C (Cryomed CryoPlus II, Thermo Fisher Scientific, Waltham, MA). Cells were stored for at least one month prior to recovery and evaluation. To evaluate cells post-thaw, vials were retrieved from storage and immediately thawed in a 37°C water bath. Vials were agitated in the bath during thaw, and removed as the last of the ice melted. The cells were then carefully washed to remove the cryoprotectant by slow dilution with complete medium over 10 minutes followed by centrifugation at $500 \times g$ for 5 minutes, aspiration of supernatant and resuspension in fresh complete medium. For these initial experiments, viability post-thaw was measured using a standard trypan blue dye exclusion assay which consisted of counting at least 100 cells to arrive at an actual percentage of live vs. dead cells. Non-frozen fresh cells served as a control. Data were initially analyzed using 2 way analysis of variance followed by Student's *t*-test for 1 and 1.5M Me₂SO.

Expanded DPSC: Optimal Cell Concentration—To determine whether cell concentration (total cell number per vial) had an effect on post-thaw viability, a brief experiment was performed using the CPA and concentration determined superior from the above experiment. For this, passage 3 cell lines from two separate DPSC cultures were

cryopreserved at 0.5, 1, 1.5, and 2 million cells per vial in 10% (~1.3M) Me₂SO prepared in complete medium (n = 4 vials per condition). The CPA solution was again added in a two-step process followed by a -1°C/minute cooling rate to -85°C then plunging into liquid nitrogen. Frozen cells were stored for at least one month at -196°C before retrieval and evaluation. To assess post thaw recovery, vials were retrieved from LN₂, thawed and washed as described above and evaluated using a standard trypan blue dye exclusion assay as compared to non-frozen controls.

Expanded DPSC: Optimized Storage Temperature—To determine the optimized storage temperature under the experimental conditions, one of the expanded cell cultures from the above experiment was sub-divided into 6 additional groups (each group consisted of 3 vials of 1 million cells per vial). These cells were then equilibrated as above with 10% (~1.3M) Me₂SO in complete medium and frozen as above to -85°C, then half of the vials were plunged into LN₂ at -196°C, with the remaining half left at -85°C for storage. Three vials were then retrieved after 1 week, 1 month and 6 months of storage at both temperatures, and evaluated initially using a standard trypan blue dye exclusion assay, and further with a functionality assay.

To measure functionality, time to confluence and doubling rates were measured using standard cell culture technique [7]. To determine doubling rates, 5×10^5 total cells were plated on 25cm² flasks and cultured for 7 days. At this point, cells were harvested using trypsin-EDTA solution, and counted again. The doubling rate was determined by comparing the cell concentration (cells per mL) on a log scale against time on a linear scale. Once harvested, the frozen thawed, expanded cultures were re-examined by flow cytometry as described above to ensure no change in cell specific markers due to storage at either temperature, and sub-cultured under differentiation assays as described above. All samples were compared to non-frozen control cells, both qualitatively and quantitatively where possible.

Cryopreservation of Digested Tissue—For these experiments, immediately following tooth tissue digestion as described above, the washed digest was equally divided into a control and experimental group (n = 9 teeth total). The control group was placed into culture immediately as described above, while the experimental group was cryopreserved using the procedure determined superior for cryopreservation of established cell cultures (e.g. 10% Me₂SO; -1°C/min cooling rate). Frozen digests were packaged in 2mL cryovials and stored for at least one month in LN₂.

Dental pulp digests were thawed and cultured in the same manner as expanded MSC. Vials were retrieved from liquid nitrogen and plunged into a 37°C water bath. Upon thawing, a slow 1:1 addition of complete medium was performed, followed by an equilibration step at 37°C for 5 minutes, and centrifugation. Supernatant was discarded and the pellet resuspended in fresh medium. Cells were plated in T-25 flasks and observed for growth by noting percent confluence, with medium changes taking place three times per week. After 14 to 28 days of growth, adherent cells were dissociated from flasks using 0.25% trypsin containing 1 mM EDTA and a total cell count obtained via standard hemacytometer counting methods.

Cryopreservation of Intact Teeth—Intact teeth were first cleaned as described above, and then subjected to equilibration by direct immersion into 10mL of 10% (~1.3M) Me₂SO in 15mL cryovials for 2 hours at 4°C to attempt full equilibration of the permeable tissues. These vials were then subjected to a dump-freezing process as described above, and stored in LN₂ for at least 1 month prior to recovery. In total, ten teeth (n = 10) were used in this experiment.

For recovery of cells, vials were thawed in a 37°C water bath, and the frozen-thawed teeth were transferred to 10mL of PBS for 30 minutes for dilution of the Me₂SO. Next the teeth were processed as described above to recover and digest the tissue. Cells were then plated in T-25

flasks and observed for growth, with medium changes taking place three times per week. After 28 days of growth, flasks were evaluated qualitatively for cell attachment, colony development and growth, and cells were harvested and counted. These numbers were compared to their non-frozen counterparts as controls, which were processed and counted the same way after 14 days of growth.

Cryopreservation of Isolated Tissue—These experiments were performed in a manner similar to intact tooth cryopreservation with some modifications. Teeth were initially cleaned, and then the roots of cleaned teeth were separated from the crown; the ends were clipped off and retained while the roots were split to reveal the dental pulp, which was then recovered with a curette. The sum of this tissue was then frozen in one of two groups. Group 1 was frozen using 10% (~1.3M) Me₂SO prepared w/v in complete medium, while Group 2 was frozen in a commercially available cryoprotectant containing 10% (~1.3M) Me₂SO in a serum free defined medium (Cryostor® CS-10, BioLife Solutions, Inc., Bothell, WA). For either group, tissue was equilibrated by transferring directly into 1mL of chilled medium in 2mL cryovials, and holding for 2 hours at 4°C. Following this, the tissue was frozen as described above and stored in LN₂ for at least one month prior to recovery.

For either group, thawing was performed by placing the vials into a 37°C water bath and removing as just the last bit of ice melted. Next the tissue was removed aseptically and transferred to 3mL of PBS at ambient (~22°C) temperature for Group 1 (Me₂SO in medium) or at 4°C for Group 2 (Cryostor CS-10). For either group, tissue was held for 30 minutes for this first dilution step. Next, the tissue was transferred to the enzyme bath and digested until the tissue just began to come apart (5–20 minutes). The resulting slurry was diluted and washed as described above, and cells were placed into culture, as described above. Cultures were then given a maximum of 20 days to establish growth of at least one colony. The groups were ranked in a non-parametric manner for cell growth as positive (at least one identifiable colony) or negative (no discernable colonies).

The resulting cultures were split upon 70% confluence (between 10–20 days) and at passage 3 cells were assayed via flow cytometry as described above and via cell differentiation assay, also described above, and compared to non-frozen controls both qualitatively and quantitatively where applicable.

RESULTS

Expanded DPSC: Optimal CPA and CPA Concentration

One million expanded cells from four separate DPSC cultures were frozen in various concentrations (0.5M – 1.5M) of ethylene glycol, propylene glycol, or dimethyl sulfoxide and stored in liquid nitrogen for 1 month. Pre-freeze viability was >95% for all four cell lines. Upon thawing, DPSC viability was assessed using a trypan blue exclusion assay. Two-way analysis of variance (ANOVA) was initially performed, the results of which indicated main effects of concentration and CPA significant ($p < 0.05$ for each) with no interactions. Subsequently, Student's *t*-test was performed which indicated that the viability for cells frozen in 1.0M and 1.5M Me₂SO ($90.6\% \pm 8.9\%$ and $91.0\% \pm 8.1\%$ respectively) were significantly better than the other CPAs at corresponding concentrations (Figure 1).

Expanded DPSC: Optimal Cell Concentration

The first experiment indicated that 1.0 or 1.5M Me₂SO yielded the optimal results for CPA and concentration. For this reason, ~1.3M Me₂SO prepared as a 10% v/v solution was used for the remaining experiments. Two DPSC cultures with pre-freeze viability >95% were frozen with different cell concentrations ($0.5 - 2.0 \times 10^6$) per vial ($n=4$ vials per condition). Upon

thawing, DPSC viability was again assessed using a trypan blue assay. No differences were statistically significant, with average post-thaw viability of all groups being >93% (Figure 2).

Expanded DPSC: Optimal Storage Temperature

At 1 week, 1 month or 6 months in storage at either -85°C or -196°C , cells were thawed and evaluated using the trypan blue assay and doubling times from log-phase cultures. Pre-freeze viability was >95%. Upon thawing, DPSC viability was $\geq 90\%$ for each time point at each temperature. The differences between doubling times were not statistically significant as determined by ANOVA between the two storage temperatures at each time interval nor between time intervals for each temperature (Figure 3). Recovered cells were also analyzed via flow cytometry to ensure no cell surface expression (Figure 4) differences and then further evaluated through differentiation culture. Cells stored at both temperatures for 6 months were cultured in osteogenic, adipogenic or chondrogenic differentiation medium for three weeks before staining with an alkaline phosphatase kit, oil red O and alcian blue, respectively. DPSC stored at each temperature were capable of undergoing trilineage differentiation.

Cryopreservation of Digested Tissue

Tissue from nine fresh teeth was digested and equally divided: half was immediately placed in culture; the other half was frozen in Me_2SO as described and stored in LN_2 for 1 month before the samples were thawed and cultured. Viable DPSC cultures were obtained from all nine freshly-cultured samples, and 8/9 frozen/thawed samples. However, DPSC from the frozen/thawed samples grew more slowly than from freshly-cultured samples. Due to impaired growth of the frozen/thawed cells, the cell number was counted after two weeks (14 days) for the immediate culture group and four weeks (28 days) for the frozen/thawed group (Figure 5). Twice as much culture time was required for the mean cell count for frozen/thawed samples to become statistically comparable (Student's *t*-test, $p > 0.05$).

Cryopreservation of Intact Teeth

Thawed teeth ($n = 10$) were processed as described above to liberate cells, which were then plated and cultured for 28 days. Of the whole teeth cryopreserved, after 28 days of culture, three teeth did not have any cells appearing morphologically as DPSC attached and exhibited no growth whatsoever. Five teeth yielded cells which attached to the flask but displayed limited growth, and even after the full culture period did not establish proliferating cultures. Two teeth yielded cells which attached immediately and grew with the characteristic growth rate of fresh DPSC cultures, and plates were confluent within 15 days. These lines were harvested and evaluated via flow cytometry to verify their cell surface expression as DPSC.

Cryopreservation of Isolated Tissue

For these experiments, undigested pulp was cryopreserved using either 10% Me_2SO in complete medium (Group 1, $n = 8$) or commercial CPA solution containing 10% Me_2SO (Cryostor-CS-10; Group 2, $n = 7$). Upon thawing and plating, the groups were ranked in a non-parametric manner as positive cell growth (at least one identifiable colony forming unit) or negative (no growth after 20 days of culture). Both groups exhibited rapid growth in all but one set of tissue from each group (87.5% and 85.7% positive growth respectively). All cultures approached confluence and required sub-culture within 20 days (Figure 6), and exhibited growth rates similar to freshly-cultured samples. There appeared to be no morphological or growth difference between cells derived from tissue frozen from either group. Cells from Group 2 were further evaluated through differentiation assay and readily demonstrated osteogenic, chondrogenic and adipogenic potential, indicating functional preservation post-thaw (Figure 7).

DISCUSSION

MSC and MSC like cells have now been isolated from various tissues, including bone marrow, adipose tissue, amniotic fluid, periosteum and fetal tissues [3,11,17,22]. MSC-like cells have been isolated from pathological tissues as well, such as the rheumatoid arthritic joint [15]. It has been suggested that cells with MSC-like characteristics likely reside in all postnatal organs and tissues [4]. It is therefore not surprising that a similar population is present in the tissues associated with teeth. Other groups have noted that the connective tissue of the human tooth arises from cells derived from the cranial neural crest, thus potentially identifying them as a highly-potent ectomesenchymal cell type [5]. As such, these cells have great potential for tissue engineering and tissue repair including neural regeneration.

Indeed MSC from various origins are currently under investigation for a number of therapeutic applications. In order to utilize cells for these purposes, they must be collected, processed, banked and distributed under cGTP principles. To that end, in this study efforts were taken to optimize post-thaw results from a functional standpoint while developing a protocol practical for maintaining cGTP.

First we determined optimized conditions for expanded, established cell cultures. Our group and others have previously demonstrated that post-thaw recovery of established cells was repeatable; our goal was to refine the system and gain any insights here to help with whole tissue cryopreservation. In these experiments it was determined that Me₂SO at a concentration between 1 and 1.5M was the ideal cryopreservative of the three studied (Fig. 1). It was also determined that DPSC viability after cryopreservation is not limited by the concentration of cells frozen, at least up to 2×10^6 cells/mL (Fig. 2). It was further established that DPSC can be stored at -85°C or -196°C for at least six months without loss of functionality (Fig. 3) utilizing the assays in this study. Further work using more quantitative assays may be required to determine if temperature of storage has more subtle effects on functionality of the cells.

Our previous preliminary experiments as well as those performed for this study indicated that cryopreservation of whole teeth with the goal of isolating DPSC which can be expanded and used clinically is not reliable or repeatable. The initial promise from our first study [18] was tarnished by the fact that even with modifications to potentially enhance the outcome (such as extended time of permeation of CPA and optimized post-thaw digestion techniques) we still did not achieve satisfactory results. While we were able to establish cultures from 7 of 10 whole teeth frozen, only two of those lines were readily expandable and functioned similar to freshly isolated cells.

Our initial speculation was that the ideal method to bank useful material with minimal manipulation would be a compromise between established cells and whole teeth: to perform the pulp tissue digest and then cryopreserve the initial cell suspension. However, we found that this method did not provide suitable results (Fig. 4), as while we were able to establish cultures from frozen-thawed digests using the procedure optimized for expanded cells, the cells did not proliferate at the same rate observed in their fresh counterpart cultures. It took at least twice as long in culture to yield similar cell counts, and the cells that did grow from the frozen/thawed digests did not look as morphologically robust, exhibiting frequent granulations corresponding to arrested development.

The optimized results with the least manipulation were achieved by isolating and cryopreserving the tooth pulp tissues, with digestion and culture performed post-thaw. By isolating the tissues, a greater degree of CPA penetration is achieved relative to an intact tooth. It is likely that water is more readily able to exit and enter the corresponding cells during the formation and melting of ice in tissue which is not trapped within the rigid dentin structure of the tooth. It is also likely that digesting the tissue first compromises the cell membrane structure

to some degree, as enzyme digestion undoubtedly results in some degree of membrane stress, potentially leading to greater cryoinjury. Post-thaw, the isolated pulp tissue may be partially digested by way of extracellular interstitial ice formation. The result is that a lesser enzymatic digestion is required to liberate cells, and they recover in culture much better. Cells derived from frozen/thawed tissue did not appear any different than fresh cultures (Fig. 5), and displayed the requisite surface markers and exhibited trilineage differentiation capability (Fig. 6) comparable to fresh cells.

Contemplating clinical use, where many regulatory bodies require no animal derived material used if possible in processing, we compared the results of the tissue cryopreservation experiments using medium with fetal calf serum to a commercially available defined medium (Cryostor-CS-10) specifically indicated for clinical banking, which worked equally well.

In conclusion, these results imply that minimal tooth processing may be needed for the banking of samples with no immediate plans for DPSC expansion and use, which in turn may limit costs and facilitate clinical banking of this potentially important cell type. Additionally, cells expanded in culture are readily preserved and can be stored for at least six months and likely longer at -85°C with respect to qualitative ability to differentiate in at least a trilineage manner. As methods for cryopreservation of the tissues of origin as well as the resulting stem cell populations are optimized and reproducible using the strategies outlined, ongoing and future research in this area includes cryopreservation of three-dimensional culture systems and cell-infiltrated scaffolds. Further studies investigating the fundamental cryobiology of these more complex systems will be crucial for widespread clinical use of this material in the future.

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Figure 1. Optimal CPA and concentration for expanded DPSC

Cryopreservation in Me₂SO preserves DPSC viability better than EG or PG. 10⁶ cells from four separate DPSC cultures were frozen in various concentrations of EG, PG or Me₂SO and stored in LN₂ for 1 month. Pre-freeze viability was >95%. Upon thawing, DPSC viability was assessed again using trypan blue staining and percent viability determined. All bars are means ± SEM.

*1 and 1.5M Me₂SO yielded significantly better (p<0.05) results than the other CPAs and/or concentrations, and were not significantly different from each other (p>0.05).

Figure 2. Effect of cell concentration on cryopreservation

Cells were frozen using 10% (~1.3M) Me₂SO at 0.5–2.0 × 10⁶ cells per mL in 1.8mL vials. Cultures from two tooth donors were frozen and stored in LN₂ for 1 month (n = 4 vials per group). Pre-freeze viability was >95%. Upon thawing, DPSC viability was assessed using trypan blue. No differences were statistically significant.

Figure 3. Effect of storage temperature on isolated DPSC

Long-term cryopreservation of DPSC at -85°C maintains viability similar to storage at -196°C . One DPSC culture was frozen using 10% ($\sim 1.3\text{M}$) Me_2SO for various periods before thawing. Doubling times were calculated from log-phase cultures. The differences between doubling times were not statistically significant ($p > 0.05$; error bars from standard error of the mean).

Non-Frozen Control Cells

Cells Stored at -85 °C

Cells Stored at -196 °C

Figure 4. Tooth-derived MSC lines express appropriate surface markers

Panel 1: Fresh MSC from passage 2 were stained with antibodies to the surface markers shown and analyzed by flow cytometry to ensure the cells meet ISCT minimal criteria for MSC (Cytotherapy 8:315-7, 2006); as did cells cryopreserved and stored at -85°C (Panel 2) and -196°C (Panel 3). Histograms (solid) from staining of one MSC line with isotype controls (open) are shown.

Figure 5. DPSC expansion after culture of fresh or frozen/thawed tissue digest

Tissue from nine teeth was digested and equally divided: half was cultured, the other half frozen in Me₂SO for 1 month, stored in LN₂, then thawed and cultured. The number of DPSC in culture were counted after two weeks for the immediate culture group (black bars) and four weeks for the frozen/thawed group (gray bars). Frozen thawed cells took twice as long to reach statistically comparable numbers of cells ($p > 0.05$; mean error bars from standard deviation).

Figure 6. Cells recovered from frozen thawed tissue in expansion culture

Cells retrieved from tissue that was digested post thaw exhibited identical morphology and growth characteristics as non-frozen cells. **A)** 24 hours after isolation and culture, cells are attaching; **B)** after 1 week of culture, cells are actively growing and exhibiting spindle shaped morphology; **C)** after two weeks of culture, cells were >70% confluent and ready for sub-culture.

Figure 7. Cells recovered from frozen thawed tissue in differentiation culture assay

DPSC recovered from frozen-thawed tissue expanded to passage 3 were then cultured in osteogenic (A), chondrogenic (B), or adipogenic (C) differentiation medium for three weeks before staining with an alkaline phosphatase kit, Alcian blue, and Oil red O respectively. This assay demonstrated DPSC from frozen/thawed tissue retain the capacity for multipotent differentiation.