Collection, Cryopreservation, and Characterization of Human Dental Pulp–Derived Mesenchymal Stem Cells for Banking and Clinical Use

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ABSTRACT

Recent studies have shown that mesenchymal stem cells (MSC) with the potential for cell-mediated therapies and tissue engineering applications can be isolated from extracted dental tissues. Here, we investigated the collection, processing, and cryobiological characteristics of MSC from human teeth processed under current good tissue practices (cGTP). Viable dental pulp–derived MSC (DPSC) cultures were isolated from 31 of 40 teeth examined. Of eight DPSC cultures examined more thoroughly, all expressed appropriate cell surface markers and underwent osteogenic, adipogenic, and chondrogenic differentiation in appropriate differentiation medium, thus meeting criteria to be called MSC. Viable DPSC were obtained up to 120 h postextraction. Efficient recovery of DPSC from cryopreserved intact teeth and second-passage DPSC cultures was achieved. These studies indicate that DPSC isolation is feasible for at least 5 days after tooth extraction, and imply that processing immediately after extraction may not be required for successful banking of DPSC. Further, the recovery of viable DPSC after cryopreservation of intact teeth suggests that minimal processing may be needed for the banking of samples with no immediate plans for expansion and use. These initial studies will facilitate the development of future cGTP protocols for the clinical banking of MSC.

INTRODUCTION

MESENCHYMAL STEM CELLS (MSC) are adult multipotent stem cells that are capable of differentiating into mesenchymal and nonmesenchymal tissues, such as fat, bone, cartilage, and neural cells.^{1–5} The findings that MSC can be relatively easily isolated from various tissues and

greatly expanded in culture make them of interest for their potential applications in tissue repair and regenerative medicine.^{6–10}

One tissue from which MSC have been more recently isolated is dental tissue. Multipotent MSC with the ability to undergo osteogenic, chondrogenic, and adipogenic differentiation as well as regenerate tooth-specific structures

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such as cementum have been isolated from the pulp,¹¹ periodontal ligaments,¹² and periapical follicles¹³ of adult teeth, as well as from deciduous teeth.¹⁴ Extracted human third molars (i.e., wisdom teeth) represent an easily accessible, often discarded tissue that may be a valuable source of MSC for future research and clinical applications. Several studies have demonstrated that intact periodontal ligaments¹⁵ or MSC isolated from dental pulp^{16,17} can be successfully cryopreserved with good viability and function upon thawing, suggesting that it may be feasible to bank dental pulp-derived MSC (DPSC) and/or dental tissue. These studies, however, examined only relatively small numbers of specimens.

To date, little has been published regarding practical aspects of obtaining and banking DPSC and/or dental tissues, such as (1) the rate of successfully establishing DPSC cultures, (2) how quickly after tooth extraction DPSC cultures must be initiated, and (3) the possibility of establishing DPSC cultures from frozen whole teeth. In this study, we obtained large numbers of extracted teeth and sought to determine how efficiently DPSC could be isolated from dental pulp under current good tissue practice (cGTP) standards. Because tissue processing for clinical banking is unlikely to take place immediately after tooth extraction, we investigated how soon after extraction DPSC must be isolated, and under what conditions teeth must be stored prior to processing, DPSC isolation, and cryopreservation. Finally, we explored whether extensive processing (i.e., isolation and culture of DPSC) was required prior to cryopreservation, or whether intact teeth could be frozen and banked for later DPSC isolation when needed.

MATERIALS AND METHODS

Collection and transport of extracted teeth

Extracted human third molars were obtained from a local oral-maxillofacial surgical center as discarded medical waste from patients 18 to 30 years of age. Oral surgeons removed any loose soft tissue and placed extracted teeth into sterile chilled vials containing 20 mL of one of three collection/transport solutions: HypoThermosol (HTS; BioLife Solutions, Bothell, WA), MesenCult basal medium (StemCell Technologies, Vancouver, BC, Canada), or phosphate-buffered saline (PBS; Sigma Chemical, St. Louis, MO). Teeth were transported on ice to the laboratory, where they were either processed immediately or used in a delayed processing time-course study (see Time-course experiments).

Processing and digestion of dental pulp, and initial culture of DPSC

Teeth were externally sterilized using a protocol adapted from cornea banking.¹⁸ Briefly, teeth received several washes in sterile PBS, followed by immersion in 1% povidone-iodine (PVP-I) for 2 min, immersion in 0.1% sodium thio-

sulfate in PBS for 1 min, and another wash in sterile PBS. The roots of cleaned teeth were separated from the crown to reveal the dental pulp, and the pulp was placed into an enzymatic bath consisting of type I and type II collagenase with thermolysin as the neutral protease (Vitacyte, Indianapolis, IN). Pulp was allowed to incubate at 37°C for 40 min to digest the tissue and liberate the cells.

Once digestion was complete, Mesencult complete medium (e.g., basal medium containing MSC stimulatory supplements; StemCell Technologies) was added to a final volume of 1.5× the digestion volume to neutralize the digestion enzymes. This mixture was centrifuged at 500 g for 5 min, and the supernatant aspirated. The cell pellet was resuspended in fresh Mesencult complete medium plus 0.25 µg/mL amphotericin B, 100 IU/mL penicillin-G, and 100 µg/mL streptomycin (JR Scientific, Woodland, CA). Cells were plated at an initial concentration of one tooth digest per 25 cm² flask. Culture flasks were monitored daily, and any contaminated flasks removed immediately and recorded. Noncontaminated flasks were monitored for cell growth, with medium changes taking place three times per week. After 14 days of growth, DPSC were detached using 0.25% trypsin/1 mM EDTA (Invitrogen, Carlsbad, CA), cell counts and viability were assessed using a standard trypan blue dye exclusion assay (Sigma) and hemacytometer, and the DPSC divided equally between two 75 cm² flasks. After the first passage, DPSC cultures were split when they reached 70% confluence. All tooth and cell processing was performed under cGTP guidelines as established by the U.S. Food and Drug Administration (<http://www.fda.gov/cber/rules/gtp.pdf>).

Efficiency of establishment of DPSC cultures from extracted teeth

To determine the efficiency of establishing DPSC cultures from teeth (outlined in Fig. 1), the first 40 teeth obtained were placed in PBS after extraction and processed immediately upon arrival in the laboratory. After 14 days the number of successfully established, viable cultures was determined. All DPSC cultures established in this phase of the study were expanded for 2–3 passages and cryopreserved for later cryopreservation/thawing experiments (see Cryopreservation and thawing of cultured DPSC and whole teeth). In addition, eight DPSC cultures were randomly selected for more detailed growth and differentiation studies and analysis of cell surface markers.

Time-course experiments

Next, we studied the time frame in which DPSC cultures could be established after tooth extraction; a schema of this experiment is shown in Figure 2. Subsequent extracted teeth (e.g., different teeth than those described in the initial experiments above) were randomly placed into vials containing PBS, HTS, or MesenCult basal medium. After arrival in

FIG. 1. Schema for initial tooth processing. Forty human molars were placed in PBS and processed immediately after extraction to determine the efficiency of establishing DPSC cultures. All 31 cultures established were expanded and frozen at passages 2–3 for later cryopreservation/thawing studies. Eight cultures were randomly chosen for further characterization.

FIG. 2. Schema of time-course experiments. Fifty-four teeth (different from those in Fig. 1) were placed into one of three collection/storage media and stored at 4°C for 0–120 h before processing. DPSC from each culture were counted after 14 days.

the laboratory, teeth were stored at 4°C for 0, 24, 48, 72, 96, or 120 h before processing, such that three uncontaminated cultures in each collection/transport solution were established at each time point ($n = 54$ teeth in total, or 18 teeth in each collection/transport solution). The number of DPSC obtained in each culture was enumerated after 14 days of growth.

DPSC growth and differentiation assays

To determine the growth rates of selected DPSC cultures, passage 3–4 DPSC were seeded in triplicate at 2×10^4 cells/well in six-well plates. After 72 h, cells were harvested and counted, and the fold-expansion and doubling time was calculated.

For differentiation assays, passage 3–4 DPSC 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) was used according to the manufacturer's instructions. Osteogenic cultures were stained for alkaline phosphatase using a leukocyte alkaline phosphatase staining kit (Sigma) as described.¹⁹ Adipogenic cultures were fixed in 4% paraformaldehyde (Sigma) and stained with 0.5% Oil Red O (Sigma) in isopropanol at room temperature. For chondrogenic differentiation, DPSC were cultured for 3–4 weeks in chondrogenic differentiation medium consisting of MesenCult complete medium with 10^{-8} M dexamethasone, 5 mg/mL ascorbic acid 2-phosphate, 10 mM β -glycerophosphate (all chemicals from Sigma), and 10 ng/mL transforming growth factor- β 3 (PeproTech, Rocky Hill, NJ). Chondrogenic cultures were fixed in 4% paraformaldehyde and incubated with 1% Alcian blue as described.¹⁹

Flow cytometry analyses

Antibodies to human CD73, CD90, CD105, CD34, CD45, CD11b, CD19, and class II human leukocyte antigen (HLA-DR) were obtained from BD Biosciences (San Jose, CA). Selected passage 3–5 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).

Cryopreservation and thawing of cultured DPSC and whole teeth

Cryopreservation of passage 2 DPSC from the initial DPSC culture-establishment experiment (Fig. 1) was performed using standard cell freezing methods.²⁰ A final concentration of 10% dimethyl sulfoxide (Cryoserv; Bioniche Pharma, Lake Forest, IL), approximately 1.3 M, in Mesencult basal medium was added drop-wise to DPSC suspensions containing $0.5\text{--}1.5 \times 10^6$ cells. DPSC suspensions were then cooled at $-1^\circ\text{C}/\text{min}$ to -85°C followed by plunging into liquid nitrogen. All cells were frozen in 2 mL cryovials (Corning, Lowell, MA). These same general freezing procedures were followed for the freezing of whole teeth, with two exceptions. Whole teeth were frozen in 15 mL cryogenic vials (Nalgene, Rochester, NY) and allowed to sit in cryoprotectant solution for 1 h at 4°C prior to freezing to aid in penetration of the cryoprotectant into the tissue. All cells and whole teeth were frozen for at least 1 month before thawing.

Vials of expanded low-passage DPSC were retrieved from liquid nitrogen and plunged into a 37°C water bath. Upon thawing, a slow addition of Mesencult complete medium

was performed, followed by an equilibration step at 37°C for 5 min, and centrifugation. The supernatant was discarded, the pellet resuspended in MesenCult complete medium, and a count and viability stain with trypan blue was conducted. Cells were plated in 25 cm² flasks and observed for growth. Whole teeth were also thawed in a 37°C water bath followed by a wash in sterile saline. Dental pulp was obtained and digested to make single-cell suspensions and cultured in the same manner as described above.

Statistics

All data are represented as the mean $\pm$ standard error of the mean (SEM). The data were compared using the unpaired Student's *t*-test, or Welch's unpaired *t*-test if the SEMs were significantly different, using InStat Version 3.05 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

RESULTS

Establishment and characterization of DPSC cultures

We sought to examine the cryobiologic characteristics of DPSC isolated from extracted human molars for the purpose of banking DPSC for later research and/or clinical use. Our initial study focused on determining how efficiently DPSC cultures could be established from relatively large numbers of extracted teeth, as shown in Figure 1. Of the first 40 teeth processed, vigorous cell growth was observed in 31/40 (77.5%) cultures after 14 days. We found that sterilization of teeth to prevent contamination was the primary obstacle to successfully establishing DPSC cultures: 7/40 (17.5%) cultures became contaminated with yeast despite the presence of amphotericin B in the culture medium. No cultures were lost due to bacterial contamination. We observed the lack of DPSC growth in only 2/40 (5%) cultures.

The appearance of DPSC over the first 2 weeks in culture is depicted in Figure 3. On day 1 after plating, single DPSC can be seen, along with considerable cell debris from the dental pulp digestion process. By days 4–6, distinct DPSC colonies could be identified. Most culture flasks were >50% confluent by day 10 and >70% confluent by day 14 (not shown), at which time DPSC cultures were trypsinized for subsequent cryopreservation experiments (see below) or further passage and characterization.

To ensure that the DPSC we cultured met minimal criteria to be called MSC,²¹ eight DPSC cultures (passages 3–5 at the time of testing) were characterized in more detail. First, flow cytometric analysis of accepted MSC cell surface markers was performed. Figure 4 shows flow cytometry histograms from one representative DPSC culture, and Table 1 shows the cumulative results from all eight DPSC cultures tested. In sum, all DPSC cultures exhibited $\geq 95\%$

FIG. 3. Appearance of early DPSC cultures. Appearance of one representative DPSC culture 1 day (upper left panel, 40 $\times$ magnification), 4 days (upper right panel, 20 $\times$), 6 days (lower left panel, 10 $\times$), and 10 days (lower right panel, 10 $\times$) after plating.

expression of CD73, CD90, and CD105, with $\leq 5\%$ expression of CD34, CD45, CD11b, CD19, and HLA-DR.

Next, we sought to determine the extent to which these eight DPSC cultures underwent trilineage (osteogenic, adipogenic, and chondrogenic) differentiation after 3–4 weeks of culture in the appropriate differentiation medium. Photomicrographs of differentiation cultures from one representative DPSC culture are shown in Figure 5. Together with the flow cytometry data, we conclude that our DPSC cultures meet minimal criteria to be termed MSC.

Time after tooth extraction in which DPSC can be cultured

Our next objective was to determine the time frame in which we could establish DPSC cultures after tooth extraction; we hypothesized that we could establish viable DPSC cultures for at least 48 h postextraction. Extracted teeth were deposited into one of three collection/transport solutions, placed on ice, and taken to the laboratory for timed storage or immediate processing, as outlined in Figure 2. From teeth processed immediately after arrival in the laboratory (0 h), DPSC cultures were readily established from teeth placed in each of the three collection/transport media (Fig. 6). At subsequent time points, the least DPSC growth was observed from teeth stored for ≥ 24 h in MesenCult basal medium. No statistical difference in the number of DPSC obtained after 14 days was observed from teeth stored in PBS or HTS at any time point. A significant difference in DPSC numbers was observed between teeth stored in PBS versus MesenCult at 0 h ($2.57 \pm 1.1 \times 10^6$ vs. $5.9 \pm 3.4 \times 10^5$ cells; $p = 0.039$), as well as between teeth stored in PBS versus MesenCult ($1.47 \pm 0.01 \times 10^6$ vs. $1.63 \pm 0.62 \times 10^5$ cells; $p = 0.0008$), and HTS versus MesenCult ($2.57 \pm 1.1 \times 10^6$ vs. $1.63 \pm 0.62 \times 10^5$ cells; $p = 0.025$) after 24 h of storage. However,

FIG. 4. DPSC cultures express appropriate surface markers. Passage 3–4 DPSC from eight independent cultures were stained with antibodies helpful in defining MSC²¹ and analyzed by flow cytometry. Results from one representative DPSC culture are shown. The open histograms signify staining with isotype controls, and the filled histograms represent staining with the specified surface marker antibody.

due to the rapid growth of cells after the first passage (doubling time range 12–30 h; $n = 6$ cultures), even if fewer cells were obtained in the first 14 days of culture, large numbers of DPSC could be obtained by expansion in subsequent passages.

Recovery of DPSC cultures from frozen DPSC cultures or whole teeth

Lastly, we investigated how efficiently DPSC could be recovered after cryopreservation of either passage 2 DPSC or whole intact teeth frozen for at least 1 month. Portions of the 31 DPSC cultures established in the initial study (Fig. 1) as well as 10 intact teeth were frozen for later study. Viable DPSC cultures were recovered from 31/31 frozen DPSC samples after thawing. The mean DPSC viability postthaw as determined by trypan blue staining was $89.5 \pm 5.1\%$ ($n = 5$ cultures); the remainder of the samples were observed

for growth in culture only. Further, viable DPSC cultures were established from 7/10 frozen whole teeth after thawing and processing as described above. No differences in growth, surface marker expression, or differentiation potential were observed in DPSC cultures analyzed before and after cryopreservation.

DISCUSSION

The objective of this study was threefold: to determine the efficiency of establishment of DPSC cultures from extracted human molars, to determine how long after tooth extraction DPSC could successfully be isolated, and to examine the cryopreservation of early passage DPSC or whole intact teeth for the potential establishment of a DPSC bank. We found that rapidly growing adherent cell cultures that meet minimal criteria to be called MSC (Figs. 3–5; Table 1) were established from 78% (31/40) of the first 40 teeth we studied. Next, we determined that DPSC cultures could be established for at least 120 h after tooth extraction (Fig. 6), and that viable DPSC could be recovered from 100% of cryopreserved early passage DPSC as well as a high percentage (70%) of frozen intact teeth after thawing. These data imply that extensive tissue processing immediately after extraction may not be a limiting factor for successful culture and/or banking of DPSC. This is of particular interest for the banking of samples with no immediate plans for MSC expansion and use, which in turn may facilitate banking of DPSC and limit banking costs. Of note, the tissue processing and cryopreservation described here was conducted under cGTP guidelines in a facility experienced and accredited in

TABLE 1. EXPRESSION OF SURFACE MARKERS ON EIGHT EXPANDED DPSC CULTURES

Antigen	% expression
CD73	99.7 ± 0.2
CD90	98.8 ± 0.9
CD105	95.6 ± 2.7
CD34	0.2 ± 0.3
CD45	0.1 ± 0.1
CD11b	1.4 ± 1.4
CD19	2.3 ± 1.1
HLA-DR	0.1 ± 0.1

FIG. 5. DPSC have the potential to undergo osteogenic, chondrogenic, and adipogenic differentiation. Passage 3–4 DPSC were cultured in osteogenic (A, 20 $\times$ magnification), chondrogenic (B, 10 $\times$ magnification), or adipogenic (C, 40 $\times$ magnification) differentiation medium for 3 weeks before appropriate staining as described in the “Materials and Methods” section. Eight DPSC lines were tested, and all were capable of undergoing trilineage differentiation. Shown are photographs of one representative DPSC line. Color images available online at www.liebertpub.com/ten.

cell and tissue banking, lending additional weight to the feasibility of DPSC banking.

While multiple reports have investigated the biological properties and applications of MSC derived from dental tissues,^{11–13,22–25} most of these reports utilized limited numbers of DPSC cultures in experiments, and did not systematically study the establishment of DPSC cultures from large numbers of extracted teeth. We were pleased that our initial efforts at establishing DPSC cultures were almost 80% successful. Not surprisingly, sterilization of extracted teeth was the primary factor determining the success of establishing DPSC cultures. For this study, we isolated DPSC only from the relatively isolated dental pulp, not from the roots or periodontal ligament, in an attempt to limit microbial contamination. As a starting point for this work, no antibiotics were added to the collection/transport media; antibiotics in the collection/transport medium may not be required, since we did not observe gross contamination of the collection/transport medium prior to processing, even of teeth that were processed up to 5 days after extraction (Fig. 6). The brief decontamination procedure with PVP-I and sodium thiosulfate we used¹⁸ was effective in removing bacteria, since no cultures were lost due to bacterial contamination. However, fungal (yeast) contamination was present in $\sim 18\%$ of cultures despite the presence of amphotericin B in the culture medium, indicating that some samples were seeded with yeast that survived the decontamination procedure. DPSC culture contamination with yeast appeared to be random in that we did not observe greater contamination of cultures from teeth stored for

FIG. 6. DPSC can be obtained from teeth transported in HTS, medium, or saline up to 120 h postextraction. Extracted teeth were placed in either PBS, HTS, or MesenCult basal medium and stored at 4°C for various periods until processing. DPSC lines were established from teeth stored in all three collection/transport media after as long as 120 h postextraction. Shown here are DPSC cell counts 14 days after the cultures were established. $n = 18$ teeth for each collection/transport solution, 3 teeth per each time point. * $p < 0.039$; † $p = 0.0008$.

several days prior to processing compared to those of teeth processed shortly after extraction, suggesting that donor-related factors, such as oral health and oral flora variability, may play a role in culture contamination. PVP-I has known efficacy in killing *Candida* species^{26,27} but may require higher concentrations and/or longer exposure than we used in this study. Brief (3 min or less) exposure to other topical antiseptic agents such as chlorhexidine^{28,29} and Listerine^{26,27,29} also efficiently kills oral pathogens, including yeast. Based on these data, we are currently incorporating additional topical antiseptics as part of the postextraction decontamination procedure.

A major limitation in tissue banking is the degradation of the tissue of interest between the time of harvest and cryopreservation.²⁰ Banking of DPSC would be greatly aided if DPSC isolation could be delayed for several days to allow for transport of teeth to the tissue bank for processing. To this end, we examined both the impact of time after extraction and three hypothermic collection/storage solutions on the establishment of DPSC cultures. Our data suggest that if extracted teeth will be processed immediately, PBS may be an acceptable, inexpensive, and widely available collection/transport medium (Fig. 6). The yield of passage 0 DPSC obtained after >72 h of cold storage clearly declines, but DPSC were still obtained up to 120 h postextraction. However, due to the rapid expansion of DPSC over the first five passages, substantial numbers of DPSC can be obtained from cultures containing fewer cells at passage 0, largely offsetting any decreased cell yields due to storage time.

Others have demonstrated that functional MSC can be recovered after cryopreservation, but no prior study has

focused on the efficiency of MSC retrieval from large numbers of frozen samples. We found that we could recover DPSC from all frozen early passage DPSC cultures. These thawed cultures showed no impairment of growth or differentiation capacity, consistent with prior studies of MSC from dental tissues,^{15,17,30} bone marrow,³¹ synovium,³² and adipose tissue.³³ Perhaps more important, however, is our finding that we could initiate DPSC cultures from 7/10 frozen whole teeth. Whereas whole teeth have been successfully cryopreserved and thawed for the purpose of reimplantation,^{34,35} our current finding implies that, with improvements in cryopreservation efficiency, banking of whole extracted teeth may be feasible, with multipotent DPSC cultured only when needed for clinical use.

In summary, we demonstrate here that DPSC cultures were established from ~80% of extracted human third molars processed within 24 h of extraction, and that DPSC cultures can be initiated for at least 120 h postextraction if teeth are stored at 4°C in a variety of collection/transport media. Further, DPSC were recovered from 100% of early passage DPSC cultures and from 70% of whole intact teeth frozen for at least 1 month. These data support the feasibility of banking DPSC and/or whole teeth for regenerative medicine applications. Current research efforts are directed at improving tooth/DPSC sterility and optimizing cryopreservation techniques while adhering to cGTP standards.

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