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# Dental pulp-derived stem cells (DPSC) differentiation *in vitro* into odontoblast and neuronal progenitors during cell passaging is associated with alterations in cell survival and viability

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## ABSTRACT

**Background:** Mesenchymal stem cells (MSC) can be derived from a variety of adult human tissues, including dental pulp from extracted or exfoliated teeth. Some evidence suggests differences in both the quality and quantity of the dental-pulp stem cells (DPSC) obtained from differing sources, such as primary or "baby teeth" and adult, permanent teeth. **Aim:** To evaluate the potential to obtain DPSC from intact, vital permanent teeth, using a randomized selection of active dental patients. **Methods:** DPSC were extracted, isolated, cultured and characterized using microscopy and RT-PCR analysis of extracted RNA. **Results:** DPSC isolates were derived from 30/31 (96.8%) of tissue explants using direct outgrowth (DO); mainly giving rise to uncommitted MSC progenitors with rapid doubling times (rDT, n=25/30 or 83.3%) and positive mRNA expression of MSC markers CD44, CD24, NANOG, Oct-4 and Sox2. DPSC isolates with slower doubling times (sDT, n=3/30 or 10%) and more limited differentiation potentials resembled neural or odontoblastic progenitor cells (sDT:npc or sDT:opc), expressed neural differentiation markers CD133 and □III tubulin or the odontoblastic differentiation marker, dentin sialophosphoprotein (DSPP), and had lower survival and viability rates following freezing, long-term storage and thawing. **Conclusions:** The need to identify potential sources of MSC to treat age-related illnesses in the current population makes it necessary to more fully explore the feasibility and potential of DPSCs extracted from adult human teeth for this newly developing field of regenerative medicine.

**Key words:** Dental pulp stem cells, mesenchymal stem cells, *in vitro* culture, neuronal progenitor, odontoblast

## INTRODUCTION

Mesenchymal stem cells (MSC), or multipotent progenitors, can be derived from a variety of adult human tissues, including dental pulp, which may provide a rich supply of multipotent, highly proliferative MSCs capable of regenerating a variety of human tissues including, but not limited to, bone and dental structures.[1-3] Dental-pulp derived stem cells (DPSC) can differentiate into many different lineages, including osteoblasts, chondroblasts, adipocytes, as well as vascular and neural tissues.[4,5]

Many factors influence both the quantity and quality of DPSC tissue available for storage and future clinical or bioengineering applications, as recent evidence suggests that DPSCs derived from human exfoliated deciduous teeth or primary teeth may have substantially higher growth and differentiation potential, as well as greater survival rates after freezing and storage when compared with DPSCs derived from vital, permanent adult teeth.[6-8] In fact, many commercial companies, such as National Dental Pulp Lab (ndpl.net) and GeneCell (genecell.com), now offer services to banking and provide long-term storage of dental pulp from primary teeth, with the marketing and advertising focused primarily on parents with young children in the process of tooth exfoliation.[9,10]

Other factors may also influence tissue quality and quantity, including the specific methods used to isolate, collect, concentrate and store DPSCs. For example, some research has suggested that the isolation of DPSCs by enzymatic dissociation (DPSC-ED) may produce heterogeneous populations of faster growing cells while the isolation technique of direct outgrowth (DPSC-OG) from tissue explants may give rise to largely homogenous populations, but with more limited differentiation potential.[11,12] Additionally, recent advances in cryobanking, cryopreservation, and storage of dental pulp have significantly increased the recovery and optimized long-term quality of dental pulp tissues – thereby increasing the possibility for new sources of MSCs for potential future clinical applications.[13,14] Finally, other studies have suggested that the choice of low- or no-serum media and the selection of early- versus late-passage populations for storage may mediate the differentiation potential and proliferative capacity of DPSC isolates.[15-17]

Most importantly, the recent nature of these discoveries means that for the majority of the population, the plethora of age-related diseases for which DPSC therapy could be useful can only be addressed by obtaining DPSC from vital, permanent adult teeth.[1,2,18] Despite the growing corpus of evidence suggesting the potential for clinical applications and possible therapies, much remains to be discovered about the potential of DPSC from healthy permanent dentition.[2,3,6]

Therefore, the primary purpose of this research project was to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients. A randomized selection of active patients requiring extractions were sampled and DPSC were extracted, isolated, cultured and characterized. The working hypothesis was that dental pulp isolates from permanent teeth would yield lower percentages of viable DPSCs, which would also exhibit comparatively slower growth and lower survival rates after freezing and storage.

## METHODOLOGY

### Human subjects

The protocol for this study titled “Isolation of Non-Embryonic Stem Cells from Dental Pulp” at the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-SDM) dental clinic was filed, amended, and approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#0907-3148) on February 5, 2010. In brief, subjects were randomly recruited by members of the UNLV-SDM clinic during their dental visits between February and June, 2010. Informed Consent was obtained onsite. Inclusion criteria: subjects had to be between eighteen (18) and sixty-five (65) years old and must agree to participate. In addition, all potential subjects must have sound, unrestored, vital teeth (teeth that have healthy pulp tissue), and need to have one or more extractions that are necessary for oral health, as

determined by the clinical faculty member in charge. Exclusion criteria: Any subject under eighteen (18) or over sixty-five (65) years of age, any subjects having dental extractions involving compromised pulp, and any subject that refuses to donate his or her extracted teeth.

## DPSC isolation and culture

In brief, dental pulp was extracted from the vital teeth of healthy adults who agreed to participate. The majority of the teeth included in this study were extracted due to severe periodontal disease, necessity for fabrication of complete dentures, or impaction and/or crowding (e.g., third molars), as follows.[7,9] Following extraction, teeth were placed into sterile 1X phosphate buffered saline (PBS) solution and transported to the laboratory for sectioning. The teeth were sectioned axially at the cemento-enamel junction (CEJ) using a diamond rotary disc in a dental handpiece and the dental pulp was removed with an endodontic broach. The dental pulp was then immediately placed into sterile microcentrifuge tubes containing 1X PBS solution and transferred to the laboratory for culture; any dental pulp not transferred within two hours was removed from the subsequent analysis. The tubes were pre-assigned randomly generated unique number to avoid bias. Demographic data such as age, gender, and ethnicity regarding the sample were obtained.

Subsequently, extracted dental pulp was vortexed for 10 – 30 seconds to dislodge cells and centrifuged for five (5) minutes at 2,100 relative centrifugal force (RCF) or g. Supernatant (PBS) was aspirated from the tube and dental pulp-derived cells were resuspended in 1.0 mL of RPMI-1640 medium from Hyclone (Logan, UT) with 2mM L-Glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate.[9] Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 g/mL) solution and 10% fetal bovine serum (FBS), obtained from HyClone (Logan, UT).[9] Cells were cultured in 75 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers.[9] Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio.

## Cell proliferation and doubling time

Cell proliferation assays were performed in the appropriate complete media, as described above.[9,10,13] In brief, cells reaching 70-80% confluence were trypsinized and plated 1:4 into new 75 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers and their confluence was measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany).[9,10,13] Doubling times (DT) from passages one (P1) though ten (P10) for each flask were recorded. Averages from the first five passages (P1-5), last five passages (P6-10) and overall average DT (P1-10) were then calculated. Data were analyzed and graphed using Microsoft Excel (Redmond, WA).

## Microscopy of cell morphology, survival and viability

During the process of passaging cells, small aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany).[8,9,19] Images were captured at 200X magnification with a Canon PowerShot G6 digital camera (Tokyo, Japan) and subsequently processed using Adobe Photoshop (San Jose, CA) Image Analysis tools. At each time point (d1-d3), several flasks were also processed using the Trypan stain, and live cells were enumerated using this procedure. Finally, DPSCs from each passage were also frozen for storage using a commercially available cryopreservation medium (Opti-Freeze) from Fisher Scientific (Fair Lawn, NJ), containing Dimethyl Sulfoxide (DMSO), using the procedure recommended by the manufacturer.[11,12] Following six weeks in storage at -80°C, cells were thawed, resuspended in the appropriate media, and live cells enumerated, as described above.

### RNA isolation, concentration, and yield

To determine if any cells derived from dental pulp were dental pulp stem cells (DPSC), RNA was isolated from 1.5 x $10^7$ cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the procedure recommended by the manufacturer for RT-PCR analysis, as previously described.[19,20] The concentration and purity of were determined using UV spectroscopy. The absorbance of diluted RNA samples (10 µL of RNA sample in 490 µL nuclease-free water, pH 7.0) was measured at 260 and 280 nm.[19,20] RNA purity was determined by calculating the ratio of A260:A280, which should be > 1.80.[19,20] Concentration for RNA samples was determined by the A260 reading of 1 = 40 µg/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water.[19,20] Concentration was calculated as 40 x A260 absorbance measure x dilution factor (50).[19,20] Total yield was determined by concentration x sample volume in mL.[19,20]

Example: RNA standard
A260 = 0.75
Concentration = 40 x 0.75 x 50 = 1,500 µg/Ml
Yield = 1,500 µg/mL x 1.0 mL = 1,500 □g or 1.5 mg RNA [19,20]

### Reverse-transcription polymerase chain reaction (RT-PCR)

To quantify the expression of DPSC-specific mRNA, RT-PCR was performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following mesenchymal stem cell (MSC) primers synthesized by SeqWright (Houston, TX) (Figure 1).[19,20]

One µg of template (total) RNA was used for each reaction.[21] The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C.[21] Thirty-five amplification cycles were run, consisting of 20-second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C.[21] Final extension was run for 5 minutes at 72°C.[21] Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME).[21] Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY).[21] Quantitation of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

### Statistical analysis

The differences between passages were determined using a t distribution, $p$= 0.05.[22] All samples were analyzed using two-tailed t-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed t-test.[22] As long as the sample size is at least moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses.[22]

## RESULTS

Thirty-one (31) individual dental pulp samples were collected from twenty-four different (24) UNLV-SDM patient clinic between February and June, 2010 and processed for cell culture using direct outgrowth (DPSC-OG), as described. This resulted in thirty DPSC isolates, with at least one from each patient, yielding an overall success rate greater than 95% percent (n = 30/31 or 96.8%).

### Cell proliferation and doubling time

During the initial growth phase (P0) each potential DPSC isolate reached 70% confluence or greater between 2 - 12 days. The average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line was then established and calculated,[19,20] revealing a characteristic average doubling time (DT) that varied from 2.5 to 10.25 days (Figure 2). The vast majority (n=27/30 or 90%) exhibited a rapid DT (rDT), ranging from 2.5 to 4 days, while the remainder (n=3/30 or 10%) exhibited a much slower DT (sDT) that ranged from 8 to

10.25 days. Although the DT remained fairly constant for most DPSC isolates, two cell lines exhibited a temporal decrease in DT observed between passages P6-10, resulting in an intermediate DT (iDT) of 5.4 and 6.4 days. The proliferation of iDT isolates was significantly faster than that of the sDT cells ($p = 0.04$) but this change was not sufficient to be significantly different than the rDT isolates ($p = 0.11$). To characterize and assess these potential DPSC isolates, microscopy was performed (Figure 3). This analysis revealed that cells with the fastest growth rates (rDT) had similar morphologies, which changed little during long-term passaging and culture; forming tightly-packed dense colonies of flat, spindle-shaped cells commonly associated with uncommitted progenitor cells (UCP) (Figure 3A). Both potential DPSC isolates that exhibited a temporal decrease to a more intermediate doubling time (iDT) appeared to have morphologies similar to rDT cells (Figure 3B), although cell-cell junctions and cell boundaries appeared slightly more distinct. Cells with the slowest growth rates (sDT) gradually adopted more distinctive morphologies, such as long, axon-like projections suggesting the formation of a sub-population of neuronal progenitor cells (NPC) (Figure 3C) or large, ovoid-shaped cells that appear to suggest to the formation of odontoblast progenitor cells (OPC) (Figure 3D). To provide more qualitative and quantitative assessments of these potential DPSC isolates, RNA was successfully isolated from all DPSC cell lines and RT-PCR was performed on equal concentrations of total RNA from each cell line (Figure 3E). Expression of mRNA for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates. For example; sDT:OPC isolates expressed comparatively lower levels of Oct-4, CD44, and NANOG, as well as the cell-cycle marker c-myc, also expressed the odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) but not alkaline phosphatase (ALP). In addition, sDT: NPC isolates did not express NANOG or CD44, but did express the neural progenitor markers CD133 and βIII tubulin. Finally, any change in viability during cell passaging and culture was evaluated before and after freezing and long-term storage (Figure 4). These results clearly demonstrate that viability among rDT and iDT isolates remained consistently high, although a slight non-significant drop in viability was apparent

| | |
|---|---|
| ALP FORWARD: | CACTGCGGACCATTCCCACGTCTT; |
| ALP REVERSE: | GCGCCTGGTAGTTGTTGTGAGCATA; |
| βIII TUBULIN FORWARD: | CTGCTCGCAGCTGGAGTGAG; |
| βIII TUBULIN REVERSE: | CATAAATACTGCAGGAGGGC; |
| c-myc FORWARD: | TCCAGCTTGTACCTGCAGGATCTGA; |
| c-myc REVERSE: | CCTCCAGCAGAAGGTGATCCAGACT; |
| CD24 FORWARD: | ACTCTCACTTGAAATTGGGC; |
| CD24 REVERSE: | GCACATGTTAATTACTAGTAAAGG; |
| CD44 FORWARD: | GAAAGGCATCTTATGGATGTGC; |
| CD44 REVERSE: | CTGTAGTGAAACACAACACC; |
| CD133 FORWARD: | CTCATGCTTGAGAGATCAGGC; |
| CD133 REVERSE: | CGTTGAGGAAGATGTGCACC; |
| DSPP FORWARD: | CAACCATAGAGAAAGCAAACGCG; |
| DSPP REVERSE: | TTTCTGTTGCCACTGCTGGGAC; |
| GAPDH FORWARD: | ATCTTCCAGGAGCGAGATCC; |
| GAPDH REVERSE: | ACCACTGACACGTTGGCAGT; |
| NANOG FORWARD: | GCTGAGATGCCTCACACGGAG; |
| NANOG REVERSE: | TCTGTTTCTTGACTGGGACCTTGTC; |
| Oct4 FORWARD: | TGGAGAAGGAGAAGCTGGAGCAAAA; |
| Oct4 REVERSE: | GGCAGATGGTCGTTTGGCTGAATA; |
| Sox2 FORWARD: | ATGGGCTCTGTGGTCAAGTC; |
| Sox2 REVERSE: | CCCTCCCAATTCCCTTGTAT; |

Figure 1: Mesenchymal stem cell (MSC) primers synthesized by SeqWright

following freezing, storage and thaw ($p$>0.05). Viability was slightly, but not significantly, lower among sDT isolates, however these isolates exhibited a significant

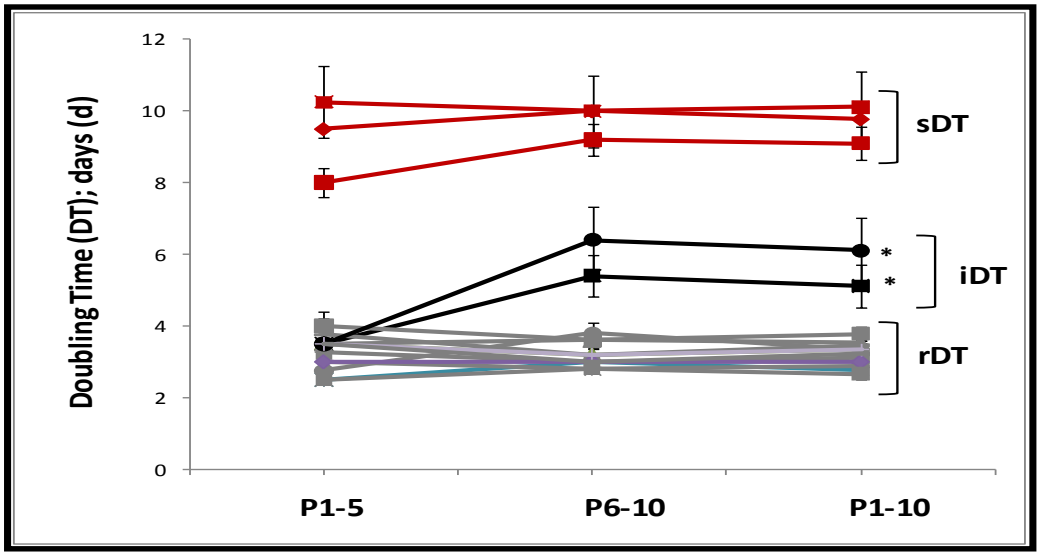


Figure 2: Cell proliferation and doubling time. Average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line varied from 2.5 to 10.25 days. Most (n=27/30 or 90%) exhibited a rapid DT (rDT): 2.5 - 4 days, while some (n=3/30 or 10%) exhibited a slower DT (sDT): 8 - 10.25 days. Two cell lines decreased DT between passages P6-10, resulting in an intermediate DT (iDT): 5.4 - 6.4 days. iDT growth was significantly faster than that of the sDT cells (p = 0.04) but not significantly different than rDT isolates ($p$ = 0.11).

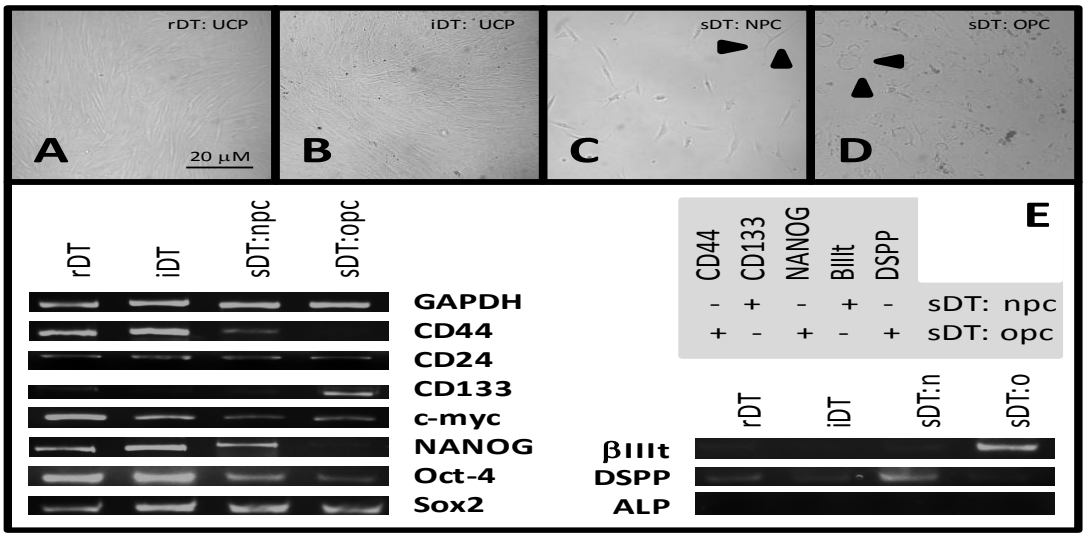


Figure 3: Microscopy and RT-PCR. A. Rapid-growth isolates (rDT) had similar morphologies; tightly-packed dense colonies of flat, spindle-shaped cells. commonly associated with uncommitted progenitor cells (UCP) (Fig. 2A). B. Cells with intermediate doubling time (iDT) exhibited morphologies similar to rDT cells. C. Slow-growth isolates (sDT) adopted distinctive morphologies, including long, axon-like projections resembling neuronal progenitor cells (NPC) or D. Large, ovoid-shaped cells resembling odontoblast progenitor cells (OPC). E. mRNA expression for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates; sDT:OPC isolates expressed comparatively lower levels of Oct-4, CD44, NANOG, and c-myc and also expressed the odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) but not alkaline phosphatase (ALP). sDT: NPC isolates did not express NANOG or CD44, but did express the neural progenitor markers CD133 and βIII tubulin.

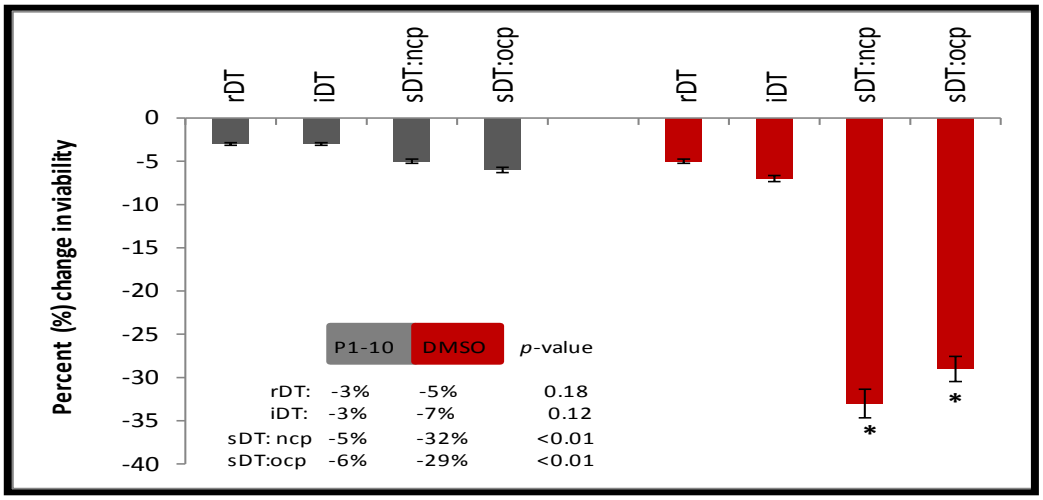


Figure 4: Viability during cell passaging and after freezing and long-term storage. Viability among rDT and iDT isolates remained high, with a small, non-significant drop in viability following freezing, storage and thaw ($p$>0.05). Viability was slightly, but not significantly, lower among sDT isolates, however these isolates exhibited a significant drop in viability following freezing, storage and thaw (sDT:ncp -32%, sDT:ocp – 29%, $p$<0.01).

drop in viability following freezing, storage and thaw (sDT:ncp -32%, sDT:ocp – 29%, $p$<0.01).

## DISCUSSION

The goals of this research project were to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients. These results clearly demonstrate the feasibility of extracting, isolating and culturing DPSC isolates from intact vital permanent teeth derived from adult patients, with a success rate exceeding 95%. In addition, although some evidence may suggest direct outgrowth (DPSC-OG) from tissue explants may give rise to slow-growing isolates with more limited differentiation potential,[9,10] these data suggest that many DPSC isolates derived from this method are, in fact, rapidly proliferating uncommitted MSC progenitors (n=25/30 or 83.3%).However, these data do confirm the previous observations that some DPSC isolates, particularly those that with slower growth rates and more limited differentiation potential (sDT:npc or opc),[11,12] are less able to survive following freezing, long-term storage and thawing for subsequent use – reducing available cells by nearly one-third.

Although many technical issues still exist, numerous researchers have elucidated how these types of uncommitted (and some partially committed) DPSC isolates may be influenced to differentiate using various methods that include cell-matrix adhesion molecules, growth factors, biomechanical scaffolding, tension and pressure.[18,23] For instance, DPSC can be induced to migrate and differentiate using extracellular matrix-coated culture materials, including Fibronectin, laminin, collagen, and fluorapatite.[24-26] Other studies have demonstrated the effects of growth factor stimulation to induce DPSCs into differentiated phenotypes, including Insulin-transferrin-sodium selenite supplement (ITS), bone morphogenic protein 2 (BMP2), growth differentiation factor 11 (Gdf11), platelet-derived growth factor (PDGF-AB), transforming growth factor (TGF-β1), dexamethasone or basic fibroblast growth factor (bFGF).[27-33] Finally, other research has demonstrated the potential to use bioscaffolding, tension and pressure to induce DPSCs toward specific differentiated phenotypes.[34-37]

## CONCLUSION

Although some commercial entities are now offering services which include processing and storage of dental pulp or DPSCs from exfoliated primary teeth or extracted third molars, less information is widely known about the viability and potential applications of DPSCs in order to provide evidence-

based recommendations for patients (and parents) interested in banking these tissues for future possible usage. The initial processing fee combined with the additional monthly storage fees – now suggests that patients, or parents of these patients, may face costs that exceed thousands of dollars for long-term storage before (or if) these cells are needed. Despite these obstacles, the need to identify potential sources of MSC for age-related illnesses in our aging population makes it necessary for dental researchers and clinicians to more fully explore the feasibility and potential for isolating and culturing DPSCs extracted from adult human teeth in order to provide more accurate and informed advice for this newly developing field of regenerative medicine.

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