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The Effects of Cryopreservation on Human Dental Pulp-Derived Mesenchymal Stem Cells

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THE EFFECTS OF CRYOPRESERVATION ON HUMAN DENTAL PULP-
DERIVED MESENCHYMAL STEM CELLS

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A thesis submitted in partial fulfillment

Of the requirements for the

Master of Science - Oral biology

School of Dental Medicine

Division of Health Sciences

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Thesis Approval

The Graduate College
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The Effects of Cryopreservation on Human Dental Pulpderived Mesenchymal Stem Cells

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Abstract

The Effects of Cryopreservation on Human Dental Pulp-Derived Mesenchymal Stem Cells

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Many studies have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions. This has driven medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies. Few studies to date have evaluated the viability of DPSC following long-term cryopreservation. The purpose of this study is to evaluate the effects of cryopreservation on dental pulp-derived stem cells (DPSC) viability over a period of three years. Dental pulp-derived stem cells were isolated and cultured from thirty-one healthy teeth. DPSC isolates were assessed for doubling-time and baseline viability prior to cryopreservation and were assessed again at three time points; one week (T1), 18 months (T2), and 36 months (T3). DPSC can be grouped based on their observed doubling times; slow (sDT), intermediate (iDT), and rapid (rDT). Viability results demonstrated all three types of DPSC isolates (sDT, iDT and rDT) exhibit time-dependent reductions in viability following cryopreservation, with the greatest reduction observed among

sDT-DPSCs and the smallest observed among the rDT-DPSC isolates. Cryopreserved DPSCs demonstrate time-dependent reductions in cellular viability. Although reductions in viability were smallest at the initial time point (T1) and greatest at the final time point (T3), these changes were markedly different among DPSC isolates with similar doubling times (DTs). Furthermore, the analysis of various DPSC biomarkers – including both intracellular and cell surface markers, revealed differential mRNA expression. More specifically, the relative high expression of Sox-2 was only found only among the rDT isolates, which was associated with the smallest reduction in viability over time. The expression of Oct4 and NANOG were also higher among rDT isolates, however, expression was comparatively lower among the sDT isolates that had the highest reduction in cellular viability over the course of this study. My second study may suggest that some biomarkers, including Nestin, NANOG, Sox-2, Oct4 may have some potential for use as biomarkers that may be associated with either higher or lower cellular viability over long-term storage applications. The analysis of these specific intracellular biomarkers revealed that Oct4 and Sox-2 may be the most important variable factors associated with both DPSC growth rate and viability during cryopreservation. This information may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers to improve both efficiency and feasibility.

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I would like to thank Dr. Karl Kingsley, my committee chair, for introducing me to this topic and for his endless time and support throughout my research topic. I would also like to thank my committee members, Dr. Cliff Seran, Dr. Ronald Lemon, and Dr. Jennifer Pharr for your support. I would like to thank my student workers, Brian Sanders and Brock Nelson, for your time and help in the laboratory.

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Chapter 1: Introduction

Background and Significance

Many recent studies using animal models have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions, including oral and maxillofacial reparation, retinal disorders, neuropathies and central nervous system disorders (Aurrekoetxea et al., 2015; Hata et al., 2015; Mead et al., 2016; Nagpal et al., 2016). Human dental pulp stem cells (DPSCs) have been shown to be very important in the future of regenerative medicine. New evidence has elucidated several potential mechanisms for inducing DPSC differentiation prior to implantation or clinical use, including induction into neural, osteogenic and odontoblastic precursors (Zhang et al., 2016; Liu et al., 2015; Ailan et al., 2015). These developments have led to considerable scientific interest in DPSC and their potential to generate novel and innovative treatments for common, as well as intractable, disease states (Collart-Dutilleul et al., 2015; Chen et al., 2015; Mead et al., 2015). These advances have driven broad medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies (Eubanks et al., 2014; Lindemann et al., 2014; Wu et al., 2015).

Storage and post thaw recovery of DPSCs techniques are very important elements to study and are key to ensure DPSCs have no loss of function and have potential to differentiate. Factors and conditions that may influence quantity and quality of the DPSCs include the specific methods used to isolate, collect, concentrate and store them, temperature stored, and length of time stored. It was shown that DPSC could be stored at -85 °C or -196 °C for at least 6 months without loss of function. Greater than 85% of DPSC were able to be recovered and isolated post-

thaw and maintained morphological and developmental competence and able to undergo differentiation (Perry et al., 2008).

Many methods have been described for DPSC cryopreservation, although no definitive standards have yet been defined for the predicted range of viability over long-term storage and the effects of differing cryopreservation methods and protocols (Perry et al., 2008; Woods et al., 2009; Gronthos et al., 2011; Gioventu et al., 2012; Lee et al., 2012). Based upon this paucity of evidence, the main objective of this study is to evaluate the effects of cryopreservation on DPSC viability over a period of three years. The current aims of this study are to characterize and evaluate the effects of long-term cryopreservation, as well as to identify biomarkers that may be useful for future potential screening and applications. These data, combined with detailed descriptions of the methods used for isolation, cryopreservation and storage will help to facilitate larger systematic reviews and meta-analyses for further evaluation of the effects of cryopreservation on the viability of DPSC during long-term storage.

Research Question

1. How does cryopreservation affect dental pulp stem cell growth? How does cryopreservation for different time frames affect viability of dental pulp stem cells? Are the survival rates of dental pulp stem cells changing through different time points?

H0: No, there is no effect on dental stem pulp cell or viability over different time frames (0 months, 18 months, 36 months) of cryopreservation.

HA: Yes; there is an effect on dental stem pulp cell or viability over different time frames (0 months, 18 months, 36 months) of cryopreservation.
2. Are there differences between the different sub-types of dental pulp stem cells (DPSCs)?

- a. What are the phenotypes of DPSC (growth)?
- b. What are the biomarkers associated with these phenotypes?
- c. What are the differences over various cryopreservation times?

H0: No, there is no difference in DPSC viability based on subtype or biomarker over different time frames (0 months, 18 months, 36 months) of cryopreservation.

HA: there is a difference in DPSC viability based on subtype or biomarker over different time frames (0 months, 18 months, 36 months) of cryopreservation.

Approval

The protocol for this study titled “Evaluation of the effects of cryopreservation on survival of dental pulp stem cells” (OPRS#763012-1) was approved by the University of Nevada, Las Vegas (UNLV) Biomedical Institutional Review Board (IRB) on August 3, 2015. The UNLV Office of Research Integrity and Protection of Research Subject (OPRS) originally approved 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; OPRS#0907-3148) on February 5, 2010. To summarize the original protocol briefly, patients were recruited at random by UNLV-SDM clinic members (faculty and students), during their dental visits between February 2010 and February 2011. Informed Consent was required and was conducted onsite.

Research Design

This research design is retrospective. Dental pulp-derived stem cells were isolated and cultured from thirty-one healthy teeth. DPSC isolates were assessed for doubling-time and

baseline viability prior to cryopreservation and will be assessed again at three time points; one week (T1), 18 months (T2), and 36 months (T3).

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Chapter 2

The Effects of Cryopreservation on Human Dental Pulp-Derived Mesenchymal Stem Cells

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Role of Authors:

Dr. Allison Tomlin designed the study, was the primary author, data collector and analyzer, and graphics generator. Michael Sanders was secondary author and assisted with data analysis and assisted with graphics generation. Dr. Karl Kingsley was tertiary author and assisted with data analysis.

Abstract

The purpose of this study is to evaluate the effects of cryopreservation on dental pulp-derived stem cells (DPSC) viability over a period of three years. Dental pulp-derived stem cells were isolated and cultured from thirty-one healthy teeth. DPSC isolates were assessed for doubling-time and baseline viability prior to cryopreservation and were assessed again at three time points; one week (T1), 18 months (T2), and 36 months (T3). DPSC can be grouped based on their observed doubling times; slow (sDT), intermediate (iDT), and rapid (rDT). Viability results demonstrated all three types of DPSC isolates (sDT, iDT and rDT) exhibit time-dependent reductions in viability following cryopreservation, with the greatest reduction observed among sDT-DPSCs and the smallest observed among the rDT-DPSC isolates. Cryopreserved DPSCs demonstrate time-dependent reductions in cellular viability. Although reductions in viability were smallest at the initial time point (T1) and greatest at the final time point (T3), these changes were markedly different among DPSC isolates with similar doubling times (DTs). Furthermore, the analysis of various DPSC biomarkers – including both

intracellular and cell surface markers, revealed differential mRNA expression. More specifically, the relative high expression of Sox-2 was only found only among the rDT isolates, which was associated with the smallest reduction in viability over time. The expression of Oct4 and NANOG were also higher among rDT isolates, however, expression was comparatively lower among the sDT isolates that had the highest reduction in cellular viability over the course of this study. These data may suggest that some biomarkers, including Sox-2, Oct4 and NANOG may have some potential for use as biomarkers that may be associated with either higher or lower cellular viability over long-term storage applications although more research will be needed to confirm these findings.

Key Words: cryopreservation, human dental pulp-derived stem cells effect

Introduction

Applications for the use of dental pulp-derived stem cells (DPSC) have received considerable attention in recent years (Potdar and Jethmalani, 2015; Conde et al., 2015). Although DPSCs may have the potential for regeneration of dental and oral tissues, recent studies have also demonstrated that DPSCs represent a novel class of mesenchymal stem cells (MSC) that may be capable of differentiation into neurons, cardioac cells, osteoblasts, as well as liver and even pancreatic cell precursors (Xiao and Nasu, 2014; Ravindran and George, 2015; Saito et al., 2015). Despite these many advances in cellular and molecular biology and bioengineering, the potential applications for DPSCs (and ultimately their clinical relevance) may be predicated upon their regenerative properties that may be dependent upon the methods used for isolation, characterization, storage and cryopreservation (Huang et al., 2009; Tatullo et al., 2014).

For example, guidelines have recently been issued by the United States regulatory agency, the American Food and Drug Administration (or FDA), as well as the European Medicines Agency (AME) for the screening and isolation of DPSC for medical-grade applications (Ducret et al., 2015). This involved using CD271-, Stro-1, and CD146-positive DPSCs frozen after P4 for 510 days, which resulted in stable post-thaw doubling times. These enhanced screening and isolation protocols may facilitate the distinction between sub-populations of DPSC with comparatively different regeneration and clinical applications, such as those expressing Stro, c-Kit, CD34, and Nestin (Ferro et al., 2014; Pisciotta et al., 2015). Although these guidelines and recommendations represent significant progress for future clinical applications, many thousands of DPSCs from clinical patients have already been isolated and stored in both commercial and academic settings, and little is known about the long-term effects of cryopreservation and storage for isolates generated prior to these new recommendations (Zhurova et al., 2010; Lindemann et al., 2014; Kumar et al., 2015).

In fact, many methods have been described for DPSC cryopreservation, although no definitive standards have yet been defined for the predicted range of viability over long-term storage and the effects of differing cryopreservation methods and protocols (Perry et al., 2008; Woods et al., 2009; Gronthos et al., 2011; Gioventu et al., 2012; Lee et al., 2012). Based upon this paucity of evidence, the main objective of this study to evaluate the effects of cryopreservation on DPSC viability over a period of three years. These data, combined with detailed descriptions of the methods used for isolation, cryopreservation and storage will help to facilitate larger systematic reviews and meta-analyses for further evaluation of the effects of cryopreservation on the viability of DPSC during long-term storage.

Methods

Human subjects

The protocol for this study titled “Evaluation of the effects of cryopreservation on survival of dental pulp stem cells” (OPRS#763012-1) was approved by the University of Nevada, Las Vegas (UNLV) Biomedical Institutional Review Board (IRB) on August 3, 2015. The UNLV Office of Research Integrity and Protection of Research Subject (OPRS) originally approved 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; OPRS#0907-3148) on February 5, 2010. To summarize the original protocol briefly, patients were recruited at random by UNLV-SDM clinic members (faculty and students), during their dental visits between February 2010 and February 2011. Informed Consent was required and was conducted 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 or other complications, 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, which were obtained mainly from the orthodontic clinic. The majority of teeth were

obtained from the orthodontic clinic, which were extracted due to impaction and/or crowding (e.g., third molars) or to provide spacing (premolars). The remainder came from the emergency clinic, which were extracted as a necessity for fabrication of complete dentures. Although most teeth removed in the emergency clinic are due to injury or due to severe periodontal disease, these were excluded from participation in this study. The teeth were immediately sectioned axially at the cemento-enamel junction (CEJ) using a diamond rotary disc in a dental hand piece and the dental pulp was removed with an endodontic broach.

The dental pulp was then immediately placed into sterile micro centrifuge 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. Tubes were pre-assigned a unique, randomly-generated number to prevent research bias. Demographic information regarding the sample was concurrently collected, which consisted of patient age, gender, and ethnicity, as well as tooth type.

Subsequently, the 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. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 mg/mL) solution and 10% fetal bovine serum (FBS), obtained from HyClone (Logan, UT). Cells were cultured in 75 cm² BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO₂ in humidified chambers. Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio.

Cell survival and viability

Cell confluence was measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). 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 or grid hemacytometer (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). During the initial growth phase each potential DPSC isolate reached 70% confluence or greater between 2 - 12 days. The average doubling time (DT) for the initial ten passages P1-P10 of each potential cell line was then established and calculated, revealing average DTs that varied from 2.5 to 10.25 days. Potential DPSC lines surviving through the tenth passage were then 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. For the current study, cell viability was determined following one week, eighteen months and thirty-six months. DPSC cell lines in storage at -80°C were thawed, resuspended in the appropriate media, and live cells enumerated, as described above.

Statistical analysis

The differences between DPSC isolates following cryopreservation (time points) were measured using a t distribution, $\alpha = 0.05$. 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 (Hayes, 1994). 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. The analyses involving multiple two sample t-tests have a higher probability of Type I error, leading to false rejection of the null hypothesis, H_0 . To confirm the effects observed from these experiments and minimize the possibility of Type I error, further analysis of the data was facilitated using ANOVA with SPSS (Chicago, IL) to more accurately assess relationships and statistical significance among and between groups.

RNA isolation

To biomarker mRNA expression from dental pulp stem cells (DPSC), RNA was isolated from 1.5×10^7 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. RNA concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10 μ L of RNA sample in 490 μ L nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A260:A280, which should be > 1.80 . Concentration for RNA samples was determined by the A260 reading of 1 = 40 μ g/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as $40 \times A_{260}$ absorbance measure \times dilution factor (50). Total yield was determined by concentration \times sample volume in mL.

Example: RNA standard $A_{260} = 0.75$

Concentration = $40 \times 0.75 \times 50 = 1,500 \mu\text{g/mL}$ Yield = $1,500 \mu\text{g/mL} \times 1.0 \text{ mL} = 1,500 \mu\text{g}$ or 1.5 mg RNA

RNA standard: GAPDH

RNA standards obtained from standardized control cells, human gingival fibroblasts isolated from 1.5×10^7 cells were used to establish the minimum threshold (CT) and saturation

(CS) cycles required for calibration and concentration comparisons using relative endpoint PCR (RE-PCR). GAPDH signal detection above background or CT required a minimum of ten cycles (C10), with saturation or CS observed at C50. Based upon these data, RE-PCR was performed at C30, above the lower detection limit but below the saturation limit.

GAPDH forward primer, 5'-ATCTTCCAGGAGCGAGATCC-3'; GAPDH reverse primer, 5'-ACCACTGACACGTTGGCAGT-3'

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):

CD44 forward primer, 5'-GAAAGGCATCTTATGGATGTGC-3' CD44 reverse primer, 5'-CTGTAGTGAAACACAACACC-3'

CD133 forward primer, 5'-CTCATGCTTGAGAGATCAGGC-3' CD133 reverse primer, 5'-CGTTGAGGAAGATGTGCACC-3'

NANOG forward primer, 5'-GCTGAGATGCCTCACACGGAG-3' NANOG reverse primer, 5'-TCTGTTTCTTGACTGGGACCTTGTC-3'

Oct4 forward primer, 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3' Oct4 reverse primer, 5'-GGCAGATGGTCGTTTGGCTGAATA-3'

Sox2 forward primer, 5'-ATGGGCTCTGTGGTCAAGTC-3' Sox2 reverse primer, 5'-CCCTCCCAATTCCCTTGTAT-5'

Klf4 forward primer, 5'-CGAACTCACACAGGCGAGAA-3' Klf4 reverse primer, 5'-CGGAGCGGGCGAATTT-3'

In brief, one ug of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. 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. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). 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). Quantitation of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

Results

To accurately determine the change in viability DPSC cell cultures were previously assessed prior to cryopreservation for speed of doubling time (DT) and viability as seen in Figure 1. More specifically, the average doubling time (DT) for the initial five passages was determined, revealing a characteristic average DT that varied within the range of 2.0 and 10.3 days (Figure 1A). Most DPSC isolates exhibited a very rapid doubling time (rDT, n=27/31) that ranged between 2.1 and 3.7 days – with a much smaller number of DPSC isolates exhibiting a much slower doubling time (sDT) of 8 – 10.1 days (n=3/31). Three DPSC isolates, however, exhibited a temporal decrease in DT observed between passages P6-P10, resulting in an intermediate doubling time (iDT) of 5.5 – 6.3 days. The baseline viability for these isolates was

measured prior to cryopreservation and was not significantly different between these three groups: sDT 94.7%; iDT 97%; rDT 95.7% ($p=0.1016$).

Viability was measured among these DPSC isolates following cryopreservation at three subsequent time intervals, after one week (T1), eighteen months (T2) and thirty six months (T3). These results demonstrated all three types of DPSC isolates (sDT, iDT and rDT) exhibit time-dependent reductions in viability following cryopreservation as seen in Figure 1B. More specifically, the sDT isolates exhibited an average reduction in viability from baseline of -26.7%, -43.7% and -49% at T1, T2 and T3, respectively. The iDT and rDT isolates also exhibited time-dependent reductions in viability from baseline of -6%, -27%, -36.5% (iDT) and -7.1%, -22.9%, -28.9% (rDT) at T1, T2, and T3 (Table 1).

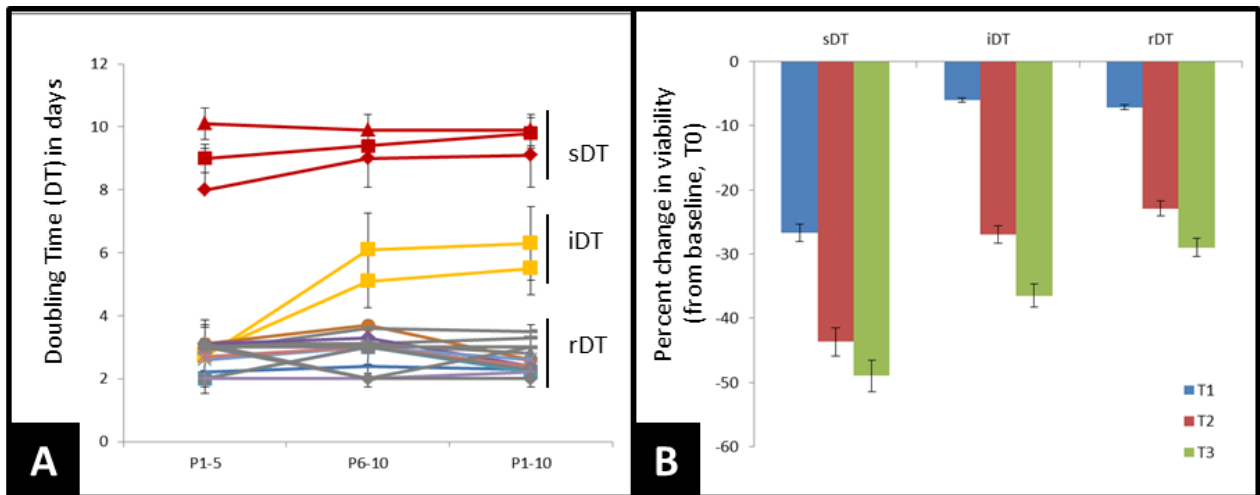


Figure 1 DPSC doubling time (DT) and viability following cryopreservation. A) Baseline estimates for proliferation or DT were determined for each of the ten passages (P10) prior to freezing and cryopreservation. Three basic groups were observed of DPSCs with rapid (rDT), intermediate (iDT) and slow (sDT) doubling times. B) Following cryopreservation, viability was

assessed at three subsequent time points; after 1 week (T1), 18 months (T2), and 36 months (T3) for comparison with baseline estimates.

Table 1 Effects of cryopreservation on DPSC viability over time

	T1 (1 week)	T2 (18 months)	T3 (36 months)
sDT	-26.7%	-43.7%	-49.0%
iDT	-6.0%	-27.0%	-36.5%
rDT	-7.1%	-22.9%	-28.9%

This demonstrated an overt difference in viability between the three types of DPSC isolates, sDT, iDT and sDT, with the greatest reduction observed among sDT-DPSCs and the smallest observed among the rDT-DPSC isolates. To more accurately assess the time-dependent trends, average DPSC isolate viability was then determined to evaluate the percent change between each time point evaluates, such as between T0 and T1 or between T1 and T2 as shown in Figure 2. These data revealed that the most striking differences between DPSC isolates was the change in viability between T0 and T1. More specifically, the reduction in viability for sDT-DPSCs was -26.7% at T1, but was similar for iDT-DPSCs (-6%,) and rDT-DPSCs (-7.1%). However, the change in viability measured from T1 to T2 was similar in all three types of DPSC isolates (-17%, -21%, -15.8%), as was the change from T2 to T3 (-5.3%, -9.5%, -6.4%).

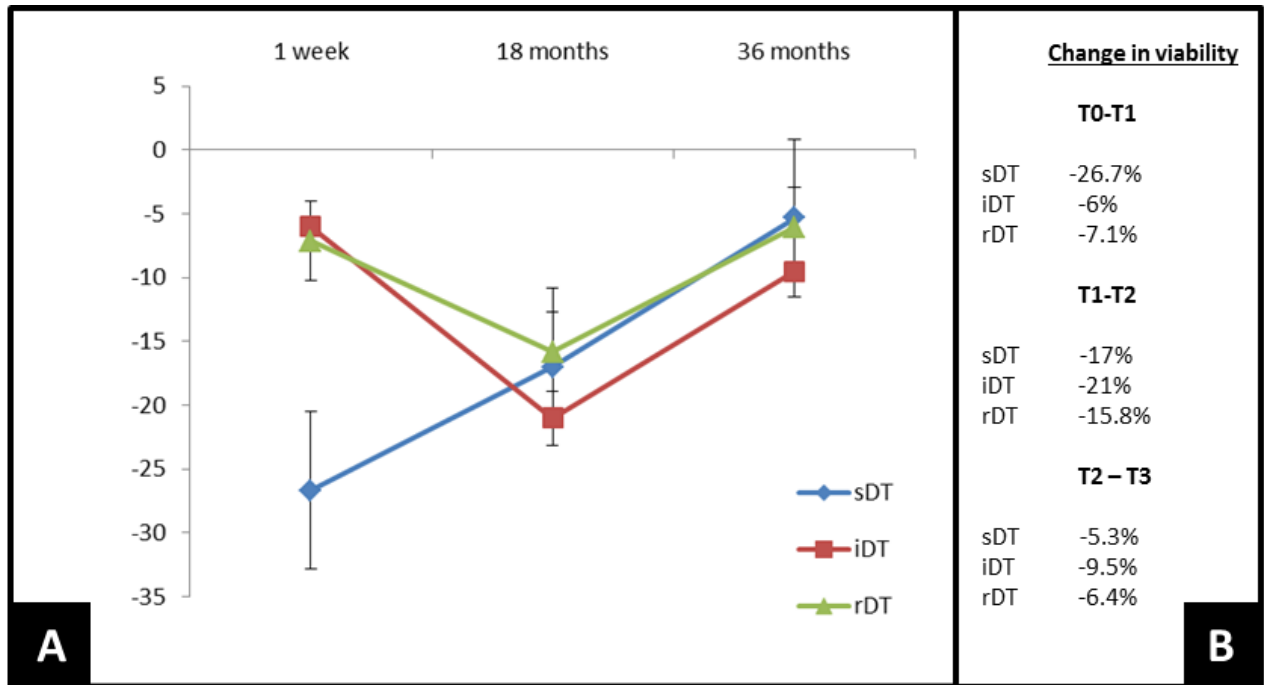


Figure 2 Analysis of viability change between time intervals following cryopreservation

The observed changes in viability for DPSC isolates with slow (sDT), intermediate (iDT) and rapid (rDT) doubling times were assessed between each time point (T0-T1, T1-T2, T2-T3), which revealed large differences in viability between T0 and T1 for sDT-DPSCs, but similar changes between T1 and T2, as well as T2 and T3 for all DPSC isolates.

In order to elucidate and evaluate these differential observations in cellular phenotype following cryopreservation, some potential factors that may contribute to these observed changes in viability over time were analyzed as seen in Figure 3. RNA was successfully isolated from all of the DPSC isolates prior to cryopreservation, which allowed for the analysis of specific intracellular biomarkers associated with DPSC in vitro including Klf, Sox2, NANOG, Oct4, as well as cell surface markers CD44 and CD133 and the housekeeping gene GAPDH (Loveland et al., 2014; Burnett et al., 2015). The original mRNA analysis was used to plot the relative intensity of the RT-bands, known as relative endpoint (RE) RT-PCR, which revealed that

expression was found to be within a narrow range for the majority of the biomarkers evaluated with some noted exceptions. These included the relatively strong expression of Sox-2 among rDT DPSC isolates, as well as the differential expression of Oct4 which was also highly expressed among rDT DPSC but had relatively low expression among sDT. In addition, NANOG expression was also markedly lower among sDT isolates. No significant differences were observed in the expression of cell surface markers or GAPDH.

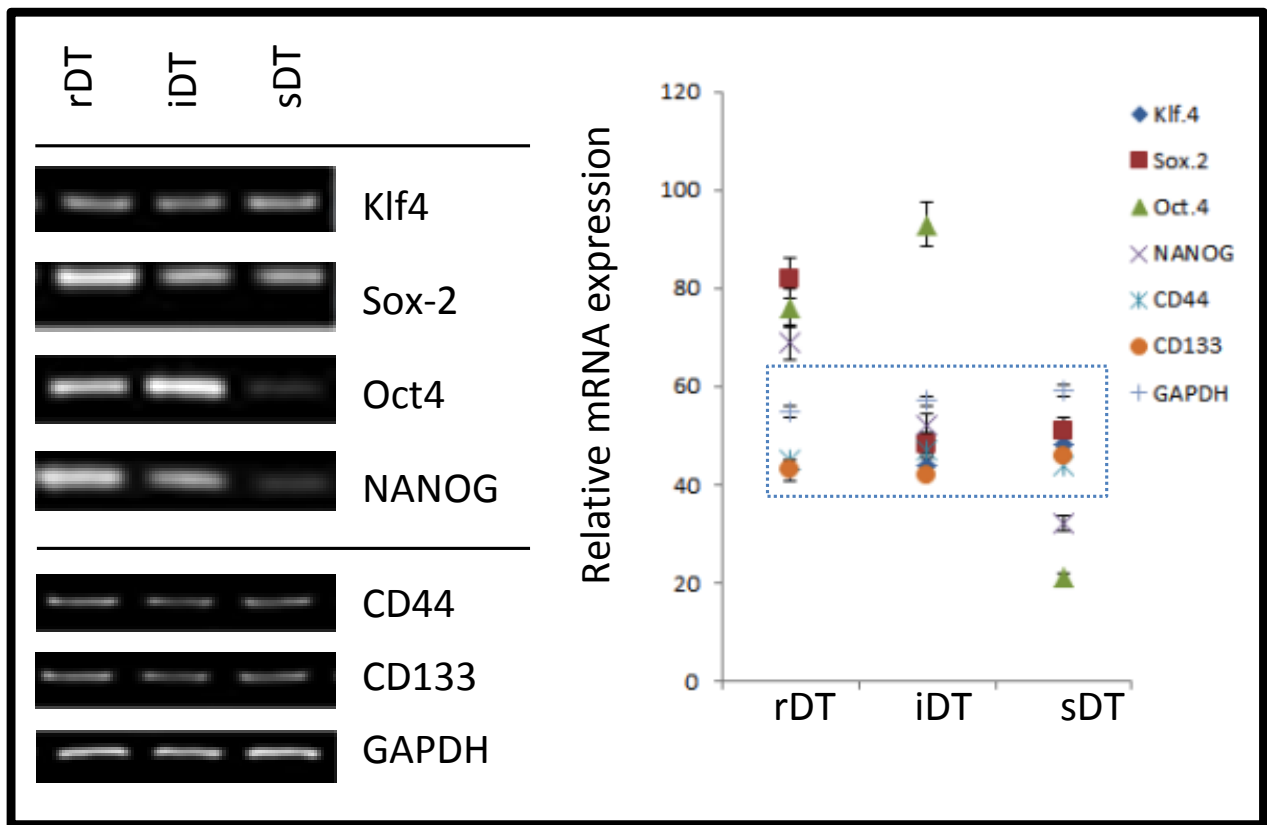


Figure 3 Analysis of mRNA expression in DPSC isolates following cryopreservation.

Discussion

The primary goal of this study was to assess the effects of cryopreservation DPSC viability over time. To further augment this analysis, initial characteristics about these DPSC

isolates were also evaluated, which included doubling time and baseline viability (Alleman et al., 2013; Hung et al., 2013). These data, combined with an evaluation of the effects of cryopreservation on the viability of DPSC during long-term storage following cryopreservation have revealed time-dependent reductions in cellular viability. Although reductions in viability were smallest at the initial time point (T1) and greatest at the final time point (T3), these changes were markedly different among DPSC isolates with similar doubling times (DTs).

For example, the reductions in viability for slowly dividing DPSC isolates (sDT, -26.7%) were higher than those observed among intermediate (iDT, -6%) or rapid (rDT, -7.1%) DPSC isolates. These data are similar to observations made in other studies of reductions to DPSC viability following cryopreservation (Xiao and Nasu, 2014; Pisciotta et al., 2015), however, these data may also reveal that some functional differences in survival may exist among DPSC isolates with varying characteristics, such as doubling time. Although these types of effects, such as reductions in cellular viability over time following cryopreservation, have been observed in other studies (Lindemann et al., 2014; Kumar et al., 2015) – this may be among the first to describe a distinguishing phenotype (doubling time) that significantly alters the viability of DPSC isolates in a more fundamental and straightforward manner.

In addition, although many other studies have described methods for optimizing cryopreservation of DPSC – these data may be among the first that categorize the viability and survival potential for DPSC isolates based upon doubling time (Perry et al., 2008; Woods et al., 2009). Although these data may be limited by the small sample size (n=31), these results may in fact reveal a more broadly applicable independent variable that can be readily and easily quantified and which may reveal that optimized methods for cryopreservation may have fundamentally differing effects on DPSC isolates with varying doubling times.

Furthermore, the analysis of various DPSC biomarkers – including both intracellular and cell surface markers, revealed most were not variable among the various isolates (Klf4, CD44, CD133 and GAPDH) although some differential expression profiles were observed among a smaller subset. More specifically, the relative high expression of Sox-2 was only found only among the rDT isolates that was associated with the smallest reduction in viability over time. Also, the expression of Oct4 and NANOG were also higher among rDT isolates – but more importantly, were found to be comparatively lower among the sDT isolates that had the highest reduction in cellular viability over the course of this study. These data may suggest that some biomarkers, including Sox-2, Oct4 and NANOG may have some potential for use as biomarkers that may be associated with either higher or lower cellular viability over long-term storage applications although more research will be needed to confirm these findings.

Conclusions

Future studies will need to explore the biomarkers and other phenotypes of rDT, iDT and sDT-DPSC isolates to determine if these baseline doubling times underlie differentiation potential or other cellular characteristics. In addition, future studies should also explore the various methods, recommendations and guidelines for isolating, characterizing, and storing DPSCs to determine if these various methods may differentially affect DPSCs with significant differences in doubling times. These data, when combined with data gleaned from other studies, provides a more thorough and comprehensive analysis of the effects of cryopreservation on DPSC isolates and may help to refine the process and ultimately the quality of clinical outcomes for future studies.

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Chapter 3

Dental pulp stem cell (DPSC) biomarker expression of Oct 4 and Sox-2 may be associated with changes to cellular viability following cryopreservation

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Role of Authors:

Dr. Allison Tomlin designed the study and was the primary author, data collector and analyzer, and graphics generator. Brock Nelson was the secondary author and assisted with data collection. Dr. Karl Kingsley was the tertiary author and assisted with data analysis and graphics generation.

Abstract

Many studies have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions, which have driven medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies. Few studies to date have evaluated the viability of DPSC following long-term cryopreservation. Based upon the paucity of information regarding long-term viability and biological markers for DPSC, the current aims of this study were to characterize and evaluate the effects of long-term cryopreservation, as well as to identify biomarkers that may be useful for future potential screening and applications. Using previously collected DPSC isolates, growth and viability over a period of four years were examined, revealing an overall decline in viability at each time point that did not appear to be linear. In addition, the analysis of specific intracellular biomarkers, including Nestin, NANOG, Sox-2 and Oct4 revealed that Oct4 and

Sox-2 may be the most important variable factors associated with both DPSC growth rate and viability during cryopreservation. This information may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers to improve both efficiency and feasibility.

Key words: cryopreservation, human dental pulp-derived stem cells, biomarker expression

Introduction

Many recent studies using animal models have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions, including oral and maxillofacial reparation, retinal disorders, neuropathies and central nervous system disorders (Aurrekoetxea et al., 2015; Hata et al., 2015; Mead et al., 2016; Nagpal et al., 2016). New evidence has elucidated several potential mechanisms for inducing DPSC differentiation prior to implantation or clinical use, including induction into neural, osteogenic and odontoblastic precursors (Zhang et al., 2016; Liu et al., 2015; Ailan et al., 2015). These developments have led to considerable scientific interest in DPSC and their potential to generate novel and innovative treatments for common, as well as intractable, disease states (Collart-Dutilleul et al., 2015; Chen et al., 2015; Mead et al., 2015).

These advances have driven broad medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies (Eubanks et al., 2014; Lindemann et al., 2014; Wu et al., 2015). For example, studies from this institution have demonstrated the feasibility and potential for the collection, isolation and in vitro mechanisms for culture-induced differentiation and de-differentiation of DPSCs (Alleman et al., 2015; Loveland et al., 2014; Burnett et al., 2015). However, despite these achievements, much remains

unknown regarding the parameters, including biological characteristics and biomarkers that influence not only differentiation, but long-term viability following extended cryopreservation (Arora et al., 2009; Gioventu et al., 2012; Ma et al., 2012).

Although some prior efforts have evaluated the effects of cryopreservation on DPSC, the majority of these studies have evaluated only short-term effects (less than six months) (Perry et al., 2008; Woods et al., 2009; Lindemann et al., 2014; Hata et al., 2015). The few studies that have investigated the effects of long-term cryopreservation and storage are providing critical knowledge towards the advancement and ultimate development of DPSC-based therapies (Ma et al., 2012; Young Kingsley, 2015; Tomlin et al., 2016). Based upon the paucity of information regarding long-term viability and biological markers for DPSC, the current aims of this study were to characterize and evaluate the effects of long-term cryopreservation, as well as to identify biomarkers that may be useful for future potential screening and applications.

Material and Methods

Human Subjects

Original approval for the collection, isolation and storage of dental pulp stem cells (DPSC) from teeth was granted for protocol OPRS#0907-3148 “Isolation of Non-Embryonic Stem Cells from Dental Pulp” in February 2010 (Alleman et al., 2013). Approval for the current study to analyze retrospectively collected biological specimens was granted for protocol OPRS#763012-1 in August, 2015. In brief, adult patients that were scheduled for an extraction in the clinic were asked to provide Informed Consent in order to participate. The majority of patient participants were had one or more healthy, vital intact teeth extracted prior to Orthodontic treatment (Hung et al., 2013). Patients having teeth extracted due to injury (fracture) or compromised dental pulp, including pulp infection or disease, were excluded.

DPSC isolation and culture

The original protocol for the collection and isolation of DPSC from vital, intact teeth involved isolation of the dental pulp from the pulp chamber following extraction. In brief, this involved cross sectioning of the tooth at the cemento-enamel junction (CEJ), following by extraction of the dental pulp with an endodontic broach which was then placed into sterile 1.5 microcentrifuge tubes with phosphate buffered saline (PBS) for transfer to the biomedical laboratory for culture. The original study protocol allowed for the isolation of dental pulp stem cells (DPSC) using the direct outgrowth method (Alleman et al., 2013; Bakopoulou et al., 2010). In brief, cells were allowed to grow for ten passages and the rate of growth or doubling time (DT) was evaluated and assessed as the interval between 1:4 passaging and achieving confluence, as previously described (Alleman et al., 2015; Loveland et al., 2014; Burnett et al., 2015; Tomlin et al., 2016). This allowed for the identification of three distinct classes of DPSC, those with rapid doubling times (rDT) less than three days, those with relatively slow doubling times (sDT) of greater than one week (8-10 days), and a smaller subset with intermediate doubling times (iDT). These phenotypes were noted for each isolate prior to cryopreservation at (-80C) using OptiFreeze Cryopreservation media from Fisher Scientific (Fair Lawn, NJ), as previously described (Alleman et al., 2015; Loveland et al., 2014; Burnett et al., 2015, Young Kingsley, 2015; Tomlin et al., 2016; Hung et al., 2013).

Cell Survival and Viability

Upon thawing at each time point (1 week, 1 month, 6 months, 12 months, 24 months, 36 months and 48 months), viability was assessed using the Trypan Blue exclusion assay as

previously described (22-24). In brief, thawed cells were centrifuged and resuspended with cell culture media RPMI-1640 with 2 mM L-Glutamine containing 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium Pyruvate, and 1% Penicillin-Streptomycin (10,000 unit/mL). Aliquots of 20 uL cell suspension were then mixed with Trypan Blue and placed into hemacytometer counting slides for analysis using a BioRad TC20 automated cell counter (Hercules, CA) using the protocol recommended by the manufacturer. These data include total cell number, total live cells (used to calculate viability) and percentage of viable cells. Three measurements were taken for each DPSC isolate for statistical analysis and averaging.

RNA isolation and RT-PCR

RNA was isolated from an aliquot of each DPSC isolate using 1.0×10^7 cells at each of the previous time points, including baseline (T0) prior to cryopreservation, and at each of the subsequent one year time points (T1-T4). RNA was isolated using the total RNA isolation reagent (TRIR) from Molecular Research Center, Inc. (Cincinnati, OH) using the protocol recommended by the manufacturer. RNA quality and quantity was assessed using spectrophotometric analysis of each sample at 260 and 280 nm. The ratio of A260:A280 measurements provides a measurement of RNA purity (acceptable range between 1.7 – 2.0) and a general estimate of quantity.

All isolates with sufficient quality (A260:A280 > 1.7) and quantity (> 1 ng/uL) were processed and screened for DPSC biomarker expression as previously described (Alleman et al., 2015; Loveland et al., 2014; Burnett et al., 2015; Young Kingsley, 2015; Tomlin et al., 2016; Hung et al., 2013). Mesenchymal stem cell (MSC) and DPSC biomarkers used in this screening included several previously validated cell surface (CD24, CD44 and CD133) and intracellular

markers (Nestin, NANOG, Sox-2, Oct4) (Liu et al., 2011, Ferro et al., 2012, Camilleri et al., 2016), as well as the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) or G3PDH, as follows:

CD24 FORWARD: ACTCTCACTTGAAATTGGGC;

CD24 REVERSE: GCACATGTTAATTACTAGTAAAGG;

CD44 forward primer, 5'-GAAAGGCATCTTATGGATGTGC-3'

CD44 reverse primer, 5'-CTGTAGTGAAACACAACACC-3'

CD133 forward primer, 5'-CTCATGCTTGAGAGATCAGGC-3'

CD133 reverse primer, 5'-CGTTGAGGAAGATGTGCACC-3'

Nestin FORWARD: CGTTGGAACAGAGGTTGGAG;

Nestin REVERSE: TCCTGAAAGCTGAGGGAAG;

NANOG forward primer, 5'-GCTGAGATGCCTCACACGGAG-3'

NANOG reverse primer, 5'-TCTGTTTCTTGACTGGGACCTTGTC-3'

Oct4 forward primer, 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3'

Oct4 reverse primer, 5'-GGCAGATGGTCGTTTGGCTGAATA-3'

Sox2 forward primer, 5'-ATGGGCTCTGTGGTCAAGTC-3'

Sox2 reverse primer, 5'-CCCTCCCAATTCCCTTGTAT-5'

GAPDH FORWARD: ATCTTCCAGGAGCGAGATCC;

GAPDH REVERSE: ACCACTGACACGTTGGCAGT

In brief, all reactions were standardized using 1 ng/uL of extracted RNA and then processed using ABgene Reverse-iT One-Step RT-PCR protocol and reagents, as previously described (Young Kingsley, 2015; Tomlin et al., 2016; Hung et al., 2013). Per standard procedures, reverse transcript was performed for 30 minutes at 47C and then 30 amplification

cycles were run, which included denaturation of 20 seconds, annealing of 30 seconds at the optimal temperature for each primer set, and five minutes of final extension at 72C. Results were visualized using gel electrophoresis and ethidium bromide in a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Rochester, NY).

Statistics

Basic descriptive statistics for viability were derived from the viability averages and reported in tables. DPSC from different categories of growth rates (rDT, iDT, sDT) were aggregated to create overall averages for these groups. Differences in viability at all time points between DPSC-rDT, -iDT, and -sDT were evaluated using two-tailed t-tests, which provide robust analysis even for samples with moderate sizes ($n \sim 20$) (Jekel et al., 2001; Glaser, 2004).

Results

All DPSC were cultured for a minimum of ten passages to establish their growth rate, which varied within the range of 2.0 and 10.3 days. The doubling times were then used to group the DPSC into rapid doubling times (rDT <3 days), intermediate doubling times (4-6 days) or comparatively slow doubling times (sDT > 8-10 days) – as previously established (Tomlin, 2016). Baseline viability was measured prior to the initial storage and cryopreservation following the initial ten passages. An aliquot from each DPSC line was retrieved from cryostorage at each of four time intervals and placed into cell culture (Figure 1). The analysis of cellular viability at each of the four time points (12 months – 48 months, T1 – T4) revealed an inverse relationship between the duration of DPSC cryopreservation and cellular viability upon thawing.

More specifically, DPSC with a rapid doubling time (rDT) exhibited an average decrease in cellular viability of -7.1% following 12 months in cryostorage, while DPSC with an intermediate doubling time (iDT) decreased an average of 6% over this time interval. DPSC with the slowest doubling time (sDT) exhibited the greatest decrease at this initial time point of -26.7%, which was statistically significant ($p < 0.01$). At each successive time point (T2-T4) all DPSC isolates exhibited decreasing viability, with the most significant declines observed between T1 and T2 – while the smallest occurred between T3 and T4.

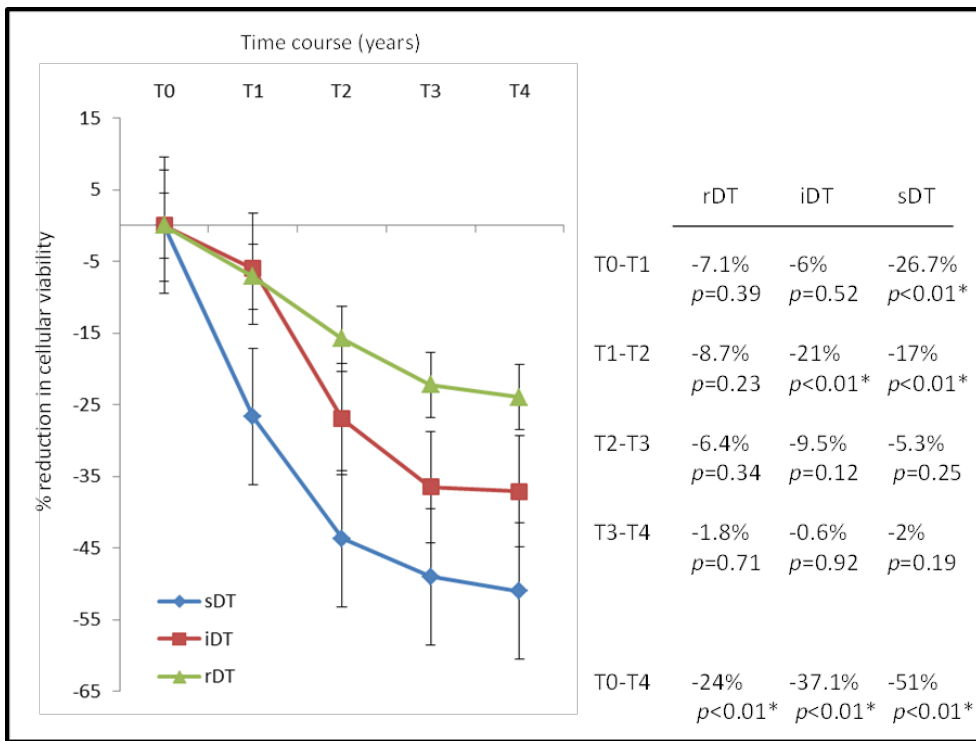


Figure 1. Effects of cryopreservation on DPSC viability. Initial viability for DPSC isolates with slow, intermediate and rapid doubling times (sDT, iDT, rDT) was compared with results following cryostorage after 12 months (T1), 24 months (T2), 36 months (T3) and 48 months (T4). This revealed overall decreased viability, which varied by DPSC type. DPSC-rDT exhibited the least reduction in viability (-24%), while DPSC-sDT exhibited the greatest reduction (-51%) ($p < 0.01$).

At each time point and determination of cellular viability, mRNA expression was assessed for multiple specific DPSC biomarkers. Some DPSC biomarkers examined (ABCG, CD24, CD44, CD133) were not included in this analysis as they exhibited no differences in mRNA expression (data not shown) (Figure 2). Intracellular mesenchymal stem cell markers Oct-4, Sox-2, NANOG and Nestin did exhibit differences in mRNA expression and were examined (Fig. 2A). This analysis revealed differential expression of mRNA among the three groups DPSC-rDT cell lines. For example, although all three groups were observed to express mRNA for Nestin and NANOG, only one DPSC-rDT expressed both Sox-2 and Oct4. The remaining DPSC-rDT exhibited differential expression of either Oct4 or Sox-2 but not both (Fig. 2B). Both of the DPSC-iDT exhibited similar mRNA expression profiles, which included Nestin, NANOG and Sox-2 but not Oct4. However, all of the DPSC-sDT exhibited similar expression of Nestin and, to a limited extent, NANOG.

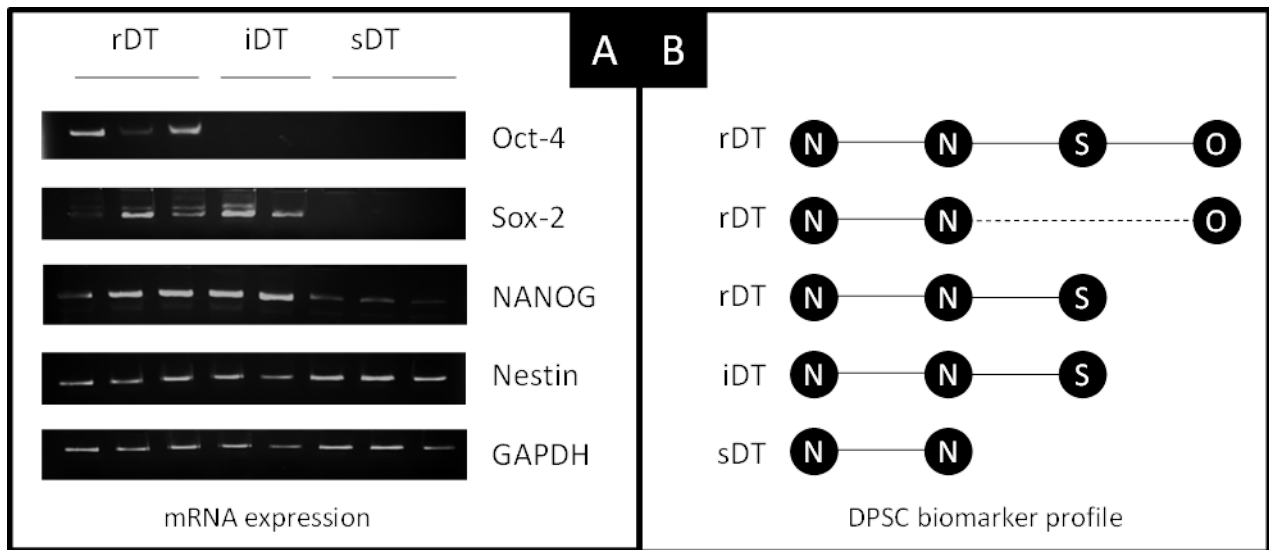


Figure 2. DPSC biomarker expression. A) Total RNA isolated from each DPSC line (rDT, iDT, sDT) was screened for expression of mRNA specific for DPSC biomarkers Oct4, Sox-2, NANOG, and Nestin – revealing differential expression among the rDT isolates. Differential expression was observed between sDT (Sox-2-, Oct4-), iDT (Oct4-) and rDT isolates. B) mRNA

expression profiles were created using these biomarkers, revealing distinct patterns specific to sDT and iDT, which may overlap with one of the rDT isolates.

Due to the resulting overlap in the DPSC biomarker expression profiles of the DPSC-iDT and one of the rDT isolates, viability of each DPSC isolate was further evaluated based upon the individual expression profile result (Figure 3). Disaggregating the rDT isolates in this analysis revealed that the rDT isolates expressing Nestin (N), NANOG (N), Sox-2 (S) and Oct4 (O) (N-N-S-O) were virtually indistinguishable from the rDT isolates that expressed Nestin, NANOG and Oct4, but not Sox-2 (N-N-O) ($p=0.668$). In addition, the overall reduction in viability for the rDT isolates that expressed Oct4 (regardless of Sox-2) expression was significantly lower than the reductions in viability among the rDT isolates that expressed Nestin, NANOG, and Sox-2 (N-N-S) but not Oct4.

Analysis of viability from the DPSC-rDT and iDT isolates with similar biomarkers expression profiles of Nestin, NANOG and Sox-2 (N-N-S) revealed similar reductions in viability at most time points, but were statistically indistinguishable from one another ($p=0.241$). Finally, the analysis of DPSC-sDT isolates, which only expressed Nestin and NANOG (N-N) revealed the greatest reduction in cellular viability at each interim time and the largest reduction overall between T0 and T4. These findings were significantly different from those of the DPSC-iDT and DPSC-rDT isolates evaluated.

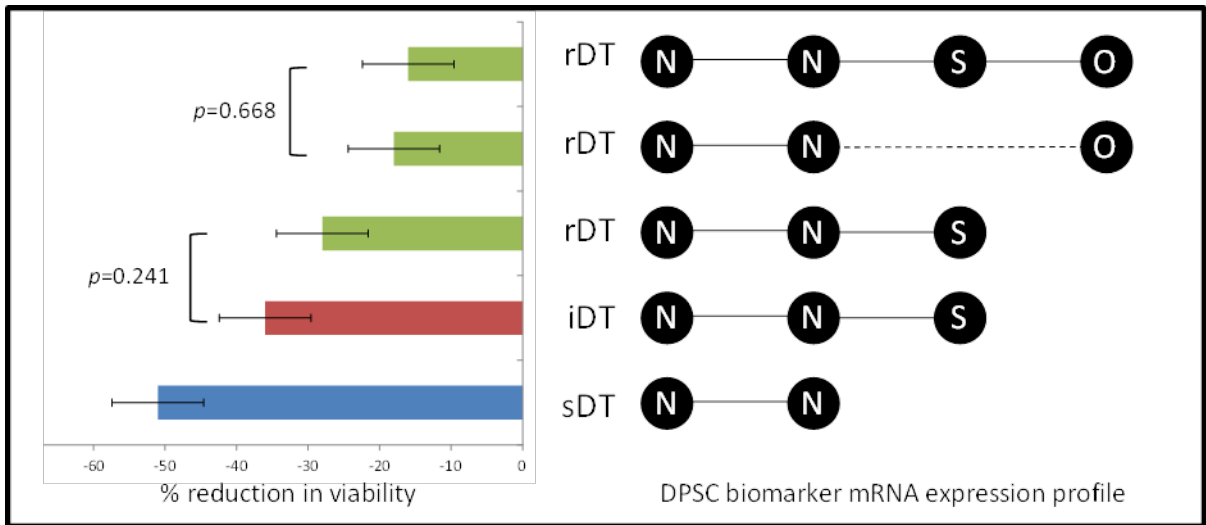


Figure 3. Analysis of cellular viability by DPSC expression profile. Using the biomarker expression profile, viability for DPSC-rDT isolates with differential expression (N-N-S-O, N-N-O, N-N-S) was compared with viability for DPSC-iDT isolates (N-N-S) revealing differential viability. Both DPSC-rDT isolates expressing Oct4 exhibited similar and smaller reductions in overall viability ($p=0.668$), while rDT and iDT with similar profiles exhibited similar, but greater reductions in viability ($p=0.241$).

Discussion

This study is among the first to provide evidence that long-term cryopreservation has significant effects on the viability of DPSC (Tomlin et al., 2016). It is important to note that although previous studies have evaluated some of the biological effects of cryopreservation on DPSC, most evaluated these effects after a period of six months or less (Hata et al., 2015; Lindemann et al., 2014; Woords et al., 2009). If clinical and therapeutic applications are to be a viable option for patients, more studies regarding the basic biology and feasibility of storage and

cryopreservation will be needed to further elucidate the parameters that govern these observations and findings.

More importantly, this study may be the first to provide evidence that the reduced viability and long-term effects of cryopreservation may not be strictly dose-dependent. For example, although some studies evaluated and analyzed viability and growth following a short time interval (usually one to two weeks) compared with a longer time interval (six months) (Hata et al., 2015; Woods et al., 2009), this study may represent the first evidence to demonstrate that the declines in viability appear to be most striking within the first two years, with smaller changes observed in following years and almost no change in viability between years three and four – regardless of DPSC phenotype (sDT, iDT, rDT). Moreover, the magnitude of these changes in viability appeared to correlate with cellular phenotype or growth rate – the more rapidly growing DPSC-rDT exhibiting the smallest reduction in viability at all time points and the slowest growth DPSC-sDT exhibiting the largest overall reduction.

To more fully examine these observations, the evaluation of biomarkers from each DPSC isolate revealed similar expression of cell surface markers (CD24, CD44, CD133) but striking differential expression of key intracellular biomarkers (NANOG, Sox-2, Oct4) (Tomlin et al., 2016; Liu et al., 2011; Ferro et al., 2012). For example, although all sDT and iDT isolates had similar expression profiles to one another (N-N and N-N-S, respectively), the three rDT isolates exhibited differential expression (N-N-S-O, N-N-O, N-N-S). Interestingly, when the viability of each individual isolate was analyzed independently, this revealed that the rDT and iDT isolates with similar biomarker profiles (N-N-S) had similar viability following cryopreservation, which was lower and distinct from the rDT that also expressed Oct4 (N-N-S-O, N-N-O). This may suggest that Oct4 but not Sox-2, both associated with pluripotency in mesenchymal and dental

pulp stem cells, may also be associated with (or an indicator of) one or more biological pathways involved in the regulation of cellular viability (Liu et al., 2011; Ferro et al., 2012).

Despite the significance of these findings, it is important to note that there are several limitations, which must also be considered. First, this is a retrospective examination of previously collected DPSC isolates – therefore, the initial conditions of isolation, culture and storage were outside the parameters of this study and could not be subjected to change or experimentation. Also, this study was conducted using patients from a public University-based dental school patient population, which may be significantly different from the traditional orthodontic patient populations seeking treatment and potential DPSC cryopreservation (Young, Kingsley 2015). Finally, differing methods or materials for cryopreservation were not studied – which may have influenced the outcomes observed in this study.

This study is among the first to provide evidence that long-term storage and cryopreservation of DPSC varies non-linear over time. This study is also among the first to provide evidence that phenotypic behaviors, such as doubling time or growth, may be one of the most important factors that determines long-term DPSC viability. Finally, this study also revealed that Oct4 and Sox-2 are among the most important variable factors that are associated with both growth and viability, which may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers.

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Chapter 4: Summary and Conclusions.

The purpose of this project was to assess the effects of cryopreservation on human dental pulp-derived mesenchymal stem cells. Chapter 2 of this document investigates DPSC viability after cryopreservation over the course of three years. Viability and doubling time assessment at multiple time points revealed the presence of 3 DPSC subtypes with varying time-dependent reductions in viability. These subtypes (sDT,iDT,rDT) all demonstrated reduction in viability over time, however, the rDT subtype demonstrated the smallest reduction in viability. The second aim of this project was to screen and characterize biomarker profiles of these subtypes and determine their correlation with survival rate. Chapter 3 describes this investigation and found distinct biomarker profiles for the DPSC isolates. Intracellular mesenchymal stem cell markers Nestin, NANOG, Sox-2, and Oct-4 presented with specific profiles for rDT, iDT, and sDT subtypes. The presence of Oct-4 biomarker was associated with the rDT subtype and greater cell viability. This suggests that Oct-4 may also be associated with pluripotency of DPSC and has involvement in the regulation of cellular viability.

This study is among the first to provide evidence that long-term storage and cryopreservation of DPSC varies non-linear over time. This study is also among the first to provide evidence that phenotypic behaviors, such as doubling time or growth, may be one of the most important factors that determines long-term DPSC viability. Finally, this study also revealed that Oct4 and Sox-2 are among the most important variable factors that are associated with both growth and viability, which may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers. This information may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers to improve both efficiency and feasibility of cryopreservation of DPSC.

Limitations and Recommendations:

One limitation to this project is that it is a retrospective examination of previously collected DPSC isolates – therefore, the initial conditions of isolation, culture and storage were outside the parameters of this study and could not be subjected to change or experimentation. This also limited the number of DPSC samples available in this study. I would recommend doing a new prospective study with new DPSC isolates to determine DPSC expressed biomarkers and growth phenotypes first and then predict changes to growth or viability over a long-term cryopreservation period in order to validate that prediction. Also, this study was conducted using samples from a public university-based dental school patient population, which may be significantly different from the traditional orthodontic patient populations seeking treatment and potential DPSC cryopreservation (Young, Kingsley 2015). It would be better to have a broader, more diverse sample size.

I would also recommend examining other alternate variables of the DPSC samples such as gender, age, and tooth type to determine if those variables affect viability and growth of DPSC. Finally, differing methods or materials for cryopreservation were not studied – which may have influenced the outcomes observed in this study. Future studies should also explore the various methods, recommendations and guidelines for isolating, characterizing, and storing DPSCs to determine if these various methods may differentially affect DPSCs with significant differences in doubling times and viability.

Appendix A



UNLV Biomedical IRB - Administrative Review Notice of Excluded Activity

DATE: August 3, 2015

TO: Karl Kingsley, PhD
FROM: UNLV Biomedical IRB

PROTOCOL TITLE: [763012-1] Evaluation of the effects of cryopreservation on survival of dental pulp stem cells.
SUBMISSION TYPE: New Project

ACTION: EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE: August 3, 2015
REVIEW TYPE: Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

Any changes to the excluded activity may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

Office of Research Integrity - Human Subjects
4505 Maryland Parkway . Box 451047 . Las Vegas, Nevada 89154-1047
(702) 895-2794 . FAX: (702) 895-0805 . IRB@unlv.edu

Appendix B

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University of Nevada, Las Vegas

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September 13, 2016

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Professor

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Title

Appendix C

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References

Chapter 1:

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