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The effects of cryopreservation on human dental pulp-derived mesenchymal stem cells

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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.

Keywords: cryopreservation; human dental pulp-derived stem cells; effect

1. Introduction

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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.

2. Materials and methods

2.1 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.

2.2 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 μg/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℃ 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.

2.3 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℃ were thawed, resuspended in the appropriate media, and live cells enumerated, as described above.

2.4 Statistical analysis

The differences between DPSC isolates following cryopreservation (time points) were measured using a t distribution, = 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.

2.5 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 uL of RNA sample in 490 uL 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 ug/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as 40 x A260 absorbance measure x dilution factor (50). Total yield was determined by concentration x sample volume in mL.
Example: RNA standard A260 = 0.75
Concentration = 40 x 0.75 x 50 = 1,500 ug/mL Yield = 1,500 ug/mL x 1.0 mL = 1,500 ug or 1.5 mg RNA

2.6 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’

2.7 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℃, followed by denaturation for 2 minutes at 94℃. Thirty-five amplification cycles were run, consisting of 20 second denaturation at 94℃, 30 seconds of annealing at 58℃, and 6.5 minutes of extension at 72℃. Final extension was run for 5 minutes at 72℃. 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.

3. 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 Fig. 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 (Fig. 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

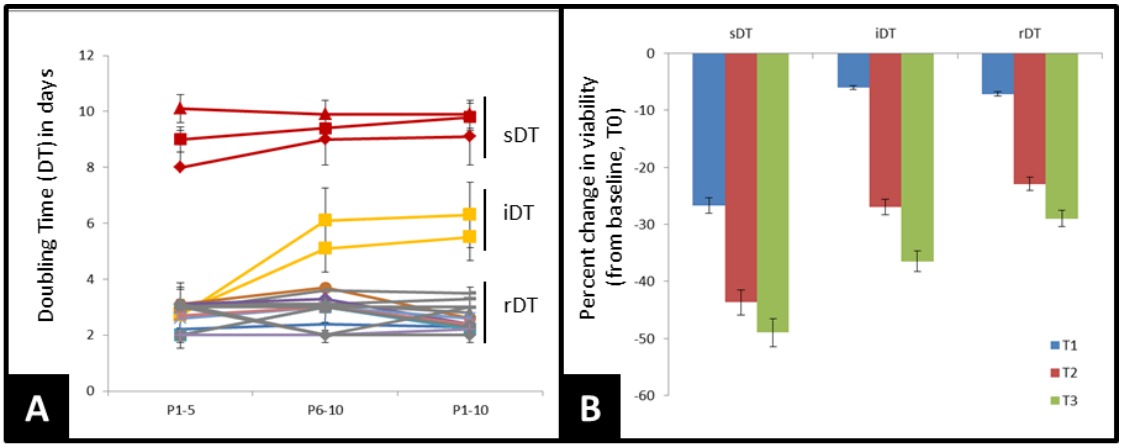

Fig. 1 DPSC doubling time (DT) and viability following cryopreservation. A) Baseline estimates for proliferation or DT were determined for each of the ten passages (P10) prior to freezing and cryopreservation. Three basic groups were observed of DPSCs with rapid (rDT), intermediate (iDT) and slow (sDT)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

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).

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%).

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

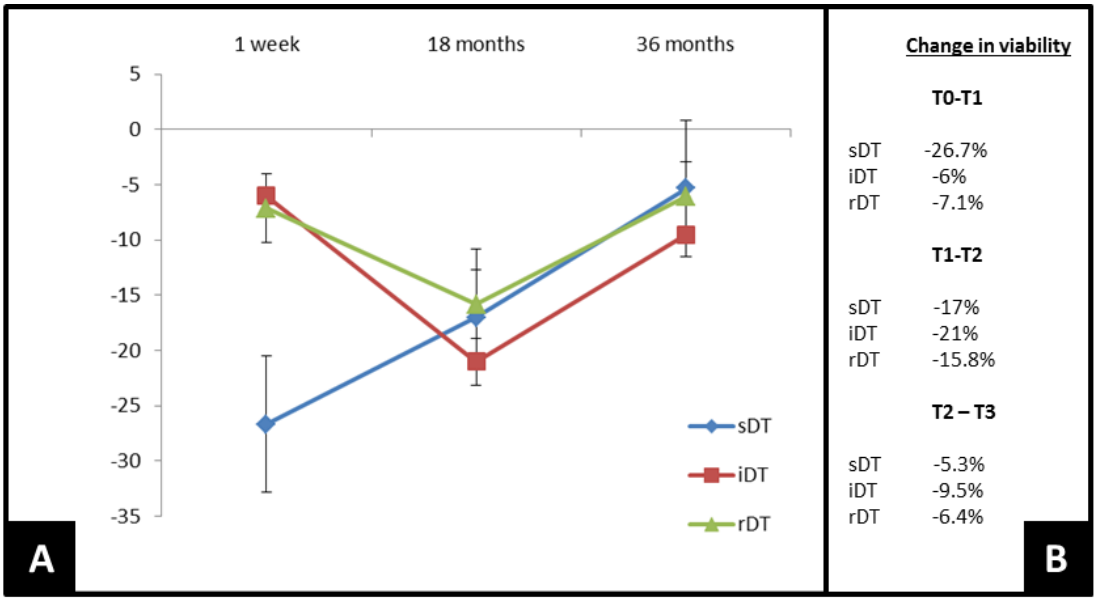

Fig. 2 Analysis of viability change between time intervals following cryopreservation

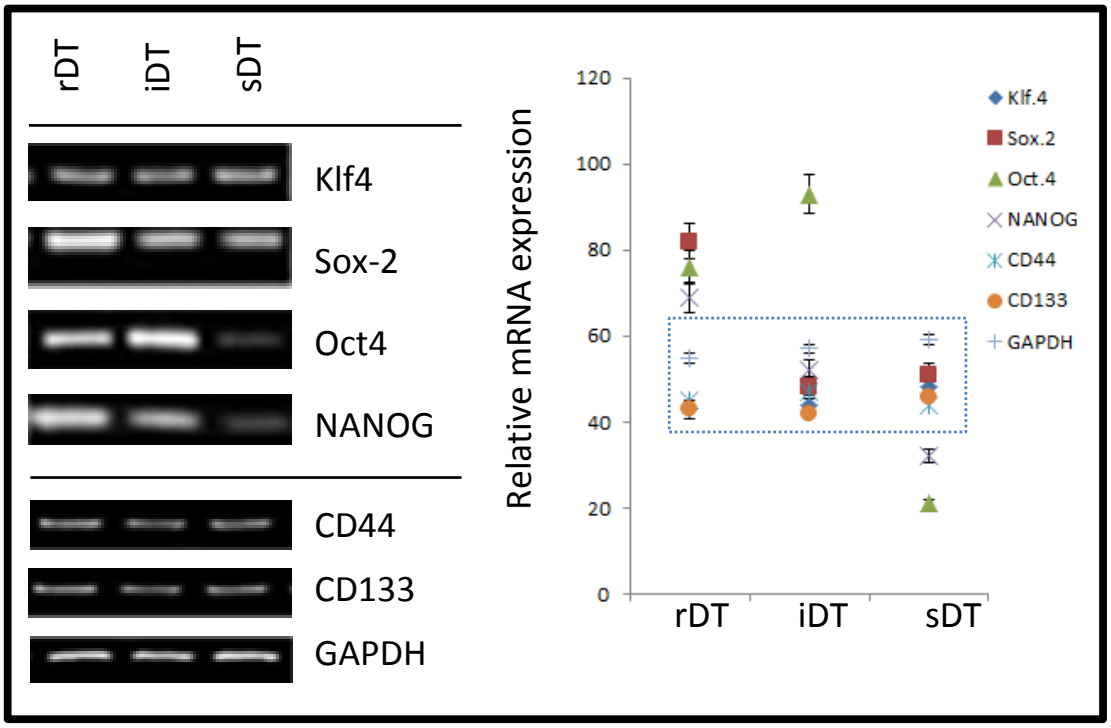

Fig. 3 Analysis of mRNA expression in DPSC isolates following cryopreservation

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 Fig. 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.

4. 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.

5. 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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