Evaluation of Polyvinylpyrrolidone as a Cryoprotectant for Adipose Tissue-Derived Adult Stem Cells

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The objective of this study was to test the hypothesis that human adipose tissue-derived adult stem cells (ASCs) can be effectively cryopreserved and stored in liquid nitrogen using a freezing medium containing a high-molecular-weight polymer, polyvinylpyrrolidone (PVP), as the cryoprotective agent (CPA) instead of dimethylsulfoxide (DMSO). To this end we investigated the postfreeze/thaw viability and apoptotic behavior of passage 1 ASCs cryopreserved in 15 different media: (i) the traditional media containing Dulbecco’s modified Eagle’s medium (DMEM) with 80% fetal calf serum (FCS) and 10% DMSO; (ii) DMEM with 80% human serum (HS) and 10% DMSO; (iii) DMEM with various concentrations (1%, 5%, 10%, 20%, and 40%) of PVP as the sole CPA; (iv) DMEM with PVP (5%, 10%, and 20%) and HS (10%); (v) DMEM with PVP (5%, 10%, and 20%) and FCS (10%); and (vi) DMEM with PVP (10%) and FCS (40% and 80%). Approximately 1 mL (10^6 cells/mL) of passage 1 ASCs were frozen overnight in a −80°C freezer and stored in liquid nitrogen for 2 weeks before being rapidly thawed in a 37°C water bath (1–2 min of agitation), resuspended in culture media, and seeded in separate wells of a six-well plate for a 24-h incubation period at 37°C. After 24 h, the thawed samples were analyzed by bright-field microscopy and flow cytometry. The results suggest that the absence of DMSO significantly increases the fraction of apoptotic and/or necrotic ASCs. However, the percentage of viable cells obtained with 10% PVP and DMEM was comparable with that obtained in freezing media with DMSO and serum (HS or FCS), that is, ~70% ± 8% and ~83% ± 8%, respectively. Slightly enhanced cell viability was observed with the addition of serum (either HS or FCS) to the freezing media containing PVP as the CPA. Adipogenic and osteogenic differentiation behaviors of the frozen thawed cells were also assessed using histochemical staining and optical density measurements and the expression of adipogenic-associated genes was analyzed using reverse transcription–polymerase chain reaction. Our results suggest that after thawing, ASC viability and adipogenic and osteogenic differentiabilities can be maintained even when ASCs are frozen in the absence of serum but with 10% PVP in DMEM.

Introduction

Developing effective techniques for the cryopreservation of human adipose tissue-derived adult stem cells (ASCs) could increase the usefulness of these cells in tissue engineering and regenerative medicine. However, for clinical applications, a large number of frozen ASCs stored under current good manufacturing practice (cGMP) will be required. Thus, an essential requirement is the development and usage of cGMP-grade reagents that are free of serum proteins and toxic chemicals because animal serum can be an undesired source of xenogenic antigens and bears the risk of transmitting animal viral, prion, and zoonose contaminations. In contrast to animal serum, the use of autologous serum eliminates the risk of infectious disease transmission. A drawback, however, is that the production of autologous serum is costly and requires a preoperative blood donation by the patient. But, to date, little attention has been paid to the development of a serum-free cryopreservation media for ASCs. This may be, in part, due to the fact that the frequency of mesenchymal stromal cells in many tissues is relatively low and the cells stored in serum-free conditions do eventually have to be expanded ex vivo. As adipose tissue can yield significantly larger number of...
ASCs,10,11 it is feasible to store large quantities of autologous ASCs derived from the patient’s own fat in serum-free conditions, so that for any potential future use these cells can be directly transferred to the clinics without any further ex vivo expansion. Indeed, published work has shown that serum-free solutions that support cells and tissues at cryopreservation temperatures can be designed.7,12–14

At present, clinically relevant survival rates for ASCs are only achieved through the use of cryopreservation solution containing animal serum and/or proteins plus a cryoprotective agent (CPA).15–19 Currently, most ASC cryopreservation protocols use the plasma membrane-permeating molecule dimethylsulfoxide (DMSO) as the CPA.15–19 Although DMSO is regarded as relatively nontoxic, the clinical use of frozen–thawed cells treated with DMSO can cause many adverse effects and toxic reactions.20–24 Therefore, for cGMP compliance, it is of particular interest to reduce the toxicity by the removal of DMSO prior to clinical use. However, the total removal of DMSO from the frozen–thawed cells is complex and time consuming.25–27 These drawbacks associated with permeating CPAs such as DMSO led us to investigate alternative freezing media for the cryopreservation of ASCs, that is, to develop or test the efficacy of freezing media without permeating cryoprotectants. There is a large divergence in choice of CPAs, ranging from low-molecular-weight solutes (permeating) such as DMSO and glycerol, to sugars such as sucrose and trehalose, and to high-molecular-weight polymers (nonpermeating) such as polyvinylpyrrolidone (PVP) and hydroxyl ethyl starch.25–35 Of direct relevance to this study, Li et al.31 showed that PVP was the sole cryoprotectant that produced maximum survival (~95%) for porcine preadipocytes. Hence, we decided to investigate the efficacy of PVP as a CPA in human ASC cryopreservation.

The objective of this study was to test the hypothesis that human ASCs can be effectively cryopreserved and stored in liquid nitrogen using a freezing medium containing PVP as the CPA instead of DMSO. To this end we investigated the postfreeze/thaw viability and apoptotic behavior of passage 1 (P1) ASCs cryopreserved in 15 different media: (i) the traditional media containing Dulbecco’s modified Eagle’s medium (DMEM) with 80% fetal calf serum (FCS) and 10% DMSO; (ii) DMEM with 80% human serum (HS) and 10% DMSO; (iii) DMEM with various concentrations (1%, 5%, 10%, 20%, and 40%) of PVP as the sole CPA; (iv) DMEM with PVP (5%, 10%, and 20%) and HS (10%); (v) DMEM with PVP (5%, 10%, and 20%) and FCS (10%); and (vi) DMEM with PVP (5%, 10%, and 40%) and FCS (40% and 80%). In our experiments the ASCs were placed in an ethanol-jacketed container which was then slowly cooled overnight in a –80°C freezer, before being transferred to liquid nitrogen. The thawed samples were analyzed by bright-field microscopy and flow cytometry to determine the percentage of viable, necrotic, and apoptotic cells. Adipogenic and osteogenic differentiation behaviors of the frozen–thawed cells were also assessed using histochemical staining and optical density (OD) measurements and the expression of adipogenic- and osteogenic-associated genes was analyzed using reverse transcription–polymerase chain reaction (RT-PCR). Our results suggest that after thawing, ASC viability and adipogenic and osteogenic differentiabilities can be maintained even when ASCs are frozen in the absence of serum but with 10% PVP in DMEM.

Materials and Methods
Isolation, collection, and culture of ASCs
All human protocols were reviewed and approved by the Pennington Biomedical Research Centre Institutional Review Board. Unless otherwise stated, all reagents were obtained from Sigma Chemicals (St. Louis, MO). Subcutaneous adipose tissue liposuction aspirates from three patients were provided by plastic surgeons in Baton Rouge, LA. These tissue samples (100–200 mL) were washed three to four times in phosphate-buffered saline (PBS), prewarmed to 37°C, suspended in PBS supplemented with 1% bovine serum albumin and 0.1% collagenase (type I; Worthington Biochemicals, Lakewood, NJ), and digested with gentle rocking for 45–60 min at 37°C. The digests were centrifuged for 5 min at 1200 rpm (300 g) at room temperature and resuspended and the centrifugation step was repeated. The supernatant was aspirated and the pellet resuspended in stromal medium (DMEM-high glucose, 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin). The cell suspension was plated at a density equivalent to 0.125 mL of liposuction tissue per square centimeter of surface area, using a 35 mL volume of stromal medium per T225 flask. The cells were cultured for 48 h in a 5% CO₂ humidified, 37°C incubator. During this time, the adherent cells were rinsed once with prewarmed PBS and the cells were fed with fresh stromal medium. The cells were fed with fresh stromal medium every 2 days until they reached ~75–80% confluence. The medium was then aspirated; the cells were rinsed with prewarmed PBS and harvested by digestion with 0.05% trypsin solution (5–8 mL per T225 flask) for 3–5 min at 37°C. The cells were suspended in stromal medium and centrifuged for 5 min at 1200 rpm (300 g), then the pellet was resuspended in a volume of 10 mL of stromal medium, and viable cell count was determined by trypan blue exclusion. These cells were identified as passage 0. The remaining cells were seeded in T225 flasks at a density of 5 × 10⁵ cells/cm². The cells were maintained in culture and passaged as described earlier to obtain P1 ASCs, which are the cells used in this study.

Preparation of freezing solutions and freezing (and thawing) experiments
The CPAs used were PVP (average molecular weight: 40,000) and DMSO (average molecular weight: 78.14). The PVP was autoclaved at 121°C for 30 min before being added to DMEM. The DMEM-PVP solutions (PVP concentration ranging from 1% to 40%) were prepared by dissolving weighted PVP in DMEM at room temperature and the solutions were then stored overnight at 4°C to obtain a homogeneous preparation. PVP concentrations above 40% were not used because the DMEM-PVP solutions become highly viscous and impossible to handle/transfer. Thus, PVP concentrations above 40% could not be (and were not) investigated.

For the freezing experiments, P1 ASCs were suspended at a concentration of 10⁶ cells/mL and equilibrated for ~10 min in the appropriate freezing media. The cell preservation fluid along with the cells was then dispensed in 2-mL cryovials, frozen overnight to –80°C in an ethanol-jacketed closed container, and subsequently stored in liquid nitrogen for further 2 weeks. The temperature/time history experienced
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by the cells in the ethanol-jacketed container was measured using type-T hypodermic needle thermocouples (Omega Technologies, Stamford, CT). Thermocouple voltages were read by a precision temperature data logger (Veriteq Instruments, Richmond, BC, Canada) and transferred to a personal computer for further reduction and data analysis. Prior to bright-field microscopy and flow cytometric analysis, individual cryovials of cells were rapidly thawed in a 37°C water bath (1–2 min of agitation), resuspended in the stromal culture media, and seeded into the separate wells of a six-well plate for a 24-h incubation period at 37°C.

**Cell viability and apoptosis/necrosis assessment**

A well-established annexin V apoptosis assay was analyzed by quantitative flow cytometry. As a chemically induced apoptotic control, ASCs were incubated in a fresh medium enriched with 40 μM etoposide for 24 h. For a necrotic control, ASCs were incubated for 24 h in a fresh medium with 5 mM hydrogen peroxide (H₂O₂). The nontreatment control consisted of ASCs treated in fresh medium, free from inducing agents. For each treatment, detached and attached cells were pooled, harvested by trypsinization (0.25% trypsin), washed with 200 μL of culture medium, and resuspended in 100 μL of 1× annexin-binding buffer (included in annexin V-FITC/propidium iodide [PI] kit). Approximately 100 μL of the cell suspension was mixed with 8 μL of annexin-V-FITC and 8 μL of 100 μg/mL PI and incubated in the dark at room temperature for 15 min. Liquid volume was removed by centrifugation and aspiration, and the cells were resuspended by gentle vortexing in 300 μL of 1× annexin-binding buffer, to be analyzed on the flow cytometer. Apoptotic analyses for ASCs were performed on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) using 488-nm laser excitation and fluorescence emission at 530 nm (FL1) and >575 nm (FL3). Forward- and side-scatter measurements were made using linear amplification, and all fluorescence measurements were made with logarithmic amplification. A total of 20,000 cells per sample were acquired using CellQuest software (BD Biosciences).

Apoptosis is characterized by phosphatidylserine translocation from the inner leaflet to the outer leaflet of the lipid bilayer, while the cell membrane remains intact. Annexin V-positive cells correspond to cells that have experienced phosphatidylserine translocation. PI staining of the cells indicates that the integrity of the cell membrane has been compromised and is used to distinguish living and early apoptotic cells from necrotic cells. The fluorescent dot plots show three quadrants: live (annexin V-FITC-negative/PI-negative), necrotic (annexin V-FITC-positive/PI-positive), and apoptotic (annexin V-FITC-positive/PI-negative). Quadrant analysis was performed on the gated fluorescent dot plot to quantify the percentage of live, necrotic, and apoptotic cell populations. The quadrants were placed according to the no-treatment control and 5 mM H₂O₂ necrotic control.

In vitro multilineage differentiation

Frozen/thawed ASCs were analyzed for their capacity to differentiate along the adipogenic lineage using the methods described below. Cell cultures of ASCs cryopreserved in 80% serum (either HS or animal serum) with 10% DMSO in DMEM were used as controls. For adipogenic differentiation, frozen-thawed ASCs were cultured in stromal media to at least 90% confluence. Adipogenesis was induced by adipogenic induction media consisting of stromal media supplemented with 1% antibiotic/antimycotic, 33 μM biotin, 17 μM pantothenate, 1 μM insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 5 μM rosiglitazone (AK Scientific, Mountain View, CA). After 3 days of induction, media were converted to adipogenesis maintenance media (identical to the induction media, except for the omissions IBMX and rosiglitazone) and cells were fed three times per week. After 14 days of maintenance, adipogenic differentiation was confirmed histologically using the oil red O stain as an indicator of intracellular lipid accumulation. To quantify lipid accumulation, the differentiated cells were washed with PBS for three to four times, fixed for 10 min in 10% formalin, and stained with 30% oil red O reagent for 60 min at room temperature. Excess stain was removed by washing with distilled water and photographs were taken of a representative field for each condition. To quantify adipogenesis, oil red O staining was eluted by 100% isopropanol and the spectrophotometric absorbance of the elution was quantified at 510 nm. Blank wells (without cells) were stained with dye and rinsed in the same manner; these values were subtracted from the experimental data points to control for stain retention by the walls of the well.

For osteogenesis, differentiation was induced after culturing frozen-thawed ASCs for 3 weeks in osteogenic media consisting of stromal media supplemented with 10 mM dexamethasone, 50 mg/mL ascorbate-2-phosphate, 10 mM 1,25 (OH)₂ vitamin D₃, and 1 M β-glycerophosphate (Bunnell et al., 2008). To determine the osteogenic potential of frozen-thawed ASCs to mineralize extracellular matrix, cells were fixed in 70% ice-cold ethanol and stained with 2% alizarin red solution (pH adjusted to 4.1–4.3) for 60 min at room temperature. To quantitate the calcium content deposited in extracellular matrix, the stain was eluted with 10% cetylpyridinium chloride for 30 min at room temperature and the OD of the solubilized stained was measured at 540 nm using a microspectrophotometer.

**Extraction of total RNA and RT-PCR analysis**

Total RNA was extracted from cells using TRI reagent according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). The cells cultured in adipogenic medium were harvested 14 days after induction and the cells cultured in osteogenic medium were harvested 21 days after induction. The cell cultures of ASCs cryopreserved in 80% FCS with 10% DMSO in DMEM were used as controls. Three adipogenic-associated genes, peroxisome proliferator-activated receptor γ2 (PPARγ2), adiponectin, and leptin, were analyzed in this study and normalized relative to cyclophilin B. The primer sets used are as follows: PPARγ (forward: AGGCCGAGGGGATCTTGT; reverse: CCCATCATAAAGGATTCTACTGATA), adiponectin (forward: GGGCGTGATGGCAAGAT; reverse: TTTCCGGATGTCTCCCTTA GG), leptin (forward: GGTGCAAGGCGCCAAGA; reverse: ACATAGAAGAGATGGGGCAAGC), and cyclophilin B (forward: GGAGATGGCACAGGAGGAAA; reverse: CGTA GTCTTCAGTTGAGTCTCA).

Real-time PCR was performed in a final reaction volume of 10 μL, including forward and reverse primers (0.1 mM),
1.5 μg reverse-transcribed RNA, and 5 μL SYBR green master mix (Applied Biosystems, Warrington, UK) using an ABI Prism 7900 instrument (Applied Biosystems, Foster City, CA). Each assay was performed in triplicate.

Statistical analysis

All values are indicated as mean ± standard error of mean. Student’s t-test was employed, with p ≤ 0.05 considered significant. ASCs derived from separate individuals were used for each replicate. Cryopreservation experiments and flow cytometry analysis were repeated six times. For flow cytometry analysis, each “n” replicate was composed of a single well and treatment in which ~20,000 cell events were analyzed and each experiment was conducted in triplicate. For adipogenic and osteogenic differentiation analyses, the staining and gene marker analysis were performed with three replicates.

Results

The various cooling rates (CRs) experienced by the cells in the ethanol-jacketed container placed in a −80 °C freezer were measured using type-T hypodermic needle thermocouples, as described earlier. The data suggest that the cells are subjected to different CRs at different time points in different freezing media within the ethanol-jacketed container. For media with 80% FCS + 10% DMSO in DMEM, the ice nucleation was observed around −8.1 ± 1.1 C, and subsequently, a CR of ~0.4 °C/min was imposed to a temperature of ~−18 °C. The CRs experienced by the cells then further drop to ~1.2 °C/min until ~−50 °C and then to ~0.4 °C/min, before reaching ~−80 °C. Alternatively, for media with 10% PVP in DMEM, there was significant super cooling before any ice nucleation was observed around −17 ± 2.3 °C. After ice nucleation, and because of the enthalpic heat release, the sample temperature was abruptly raised to ~−5 °C and a subsequent CR of ~0.4 °C/min was imposed to a temperature of ~−10 °C. The CRs experienced by the cells then further drop to ~−1.8 °C/min until ~−50 °C and then to ~0.4 °C/min, before reaching ~−80 °C.

Characteristic flow cytometer fluorescent dot plots for ASCs frozen–thawed with different concentrations of PVP as determined by annexin V staining and PI uptake are shown in Figure 1. The fluorescent dot plots for cells frozen in the presence of 1% and 40% PVP (Fig. 1A, E) show a majority of the cells in the upper right quadrant (annexin V~/~/PI~/~) which corresponds to necrotic cell population. Between 1% and 40% PVP, the number of cells present in the lower left quadrant (annexin V~/~/PI~/~), which corresponds to live cell population, initially increases and then decreases with maximum number of live cells showing at 10% PVP (Fig. 1B–D). Similar population quadrant analysis was performed on all other experimental treatments to obtain quantitative information on the condition of the cells following the freeze–thaw process (Tables 1 and 2).

FIG. 1. Characteristic flow cytometer fluorescent dot plots showing fluorescence-activated cell sorting analysis of cell death after freezing, determined by annexin V staining and PI uptake, for ASCs treated and frozen in the presence of (A) 1%, (B) 5%, (C) 10%, (D) 20%, and (E) 40% PVP, respectively. PI, propidium iodide; ASCs, adipose tissue-derived adult stem cells; PVP, polyvinylpyrrolidone. Color images available online at www.liebertonline.com/ten.
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Table 1. Effect of Control Treatments and Assay Controls on Resulting Percentages of Necrotic, Live, and Apoptotic Adipose Tissue-Derived Adult Stem Cells According to the Annexin V Apoptosis Assay Protocol

<table>
<thead>
<tr>
<th>Control treatments</th>
<th>% Viable cells (±SD)</th>
<th>% Apoptotic cells (±SD)</th>
<th>% Necrotic cells (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% DMEM + 80% HS + 10% DMSO</td>
<td>82.5 (±8.3)</td>
<td>5.8 (±3.9)</td>
<td>8.3 (±3.3)</td>
</tr>
<tr>
<td>10% DMEM + 80% FCS + 10% DMSO</td>
<td>84.1 (±7.7)</td>
<td>5.7 (±2.8)</td>
<td>7.5 (±3.7)</td>
</tr>
</tbody>
</table>

As shown in Figure 2, the viability of ASCs frozen in PVP and DMEM resembles an inverted U-shape (with 10% PVP being optimal). The use of 1% and 40% PVP in DMEM caused a dramatic decrease in cell viability (~5%), whereas the use of 10% PVP produced a maximum viability of ~70% (Table 2). The viability decreased significantly when the percentage of PVP was increased to either 20% or 40% in DMEM. A similar but opposite trend was observed for the percentage of cells exhibiting necrosis. As expected, based on the viability response, the percentage of necrotic ASCs showed a U-shaped response with increasing concentration of PVP. ASCs treated with 1% and 40% PVP treatments were found to have a significant amount of necrotic cells (~65–75%) when compared with other treatments (~12–27%). Intriguingly, the percentage of apoptotic cells decreased from ~23% to ~5% as the concentration of PVP in DMEM was increased from 1% to 40%.

Table 2 also shows the postfreeze response of ASCs frozen at three concentrations (5%, 10%, and 20%) of PVP supplemented with either 10% FCS or 10% HS in DMEM. The addition of serum to the freezing media increased the percentage of viable ASCs by ~0–9% when compared with the values obtained in their absence. However, a closer examination of the data revealed that this improvement in the postthaw cell viability was not statistically significant (95% confidence level). Similarly, the variations in the measured values of apoptotic and necrotic cells obtained in the presence of HS or FCS with PVP and DMEM when compared with the values obtained with just PVP in DMEM are also not statistically significant (95% confidence level). To further analyze the effect of increasing the percentage of serum in

Table 2. Flow Cytometric Measurements of Apoptosis and Necrosis in Various Cryopreservation Media

<table>
<thead>
<tr>
<th>Freezing media</th>
<th>% Viable cells (±SD)</th>
<th>% Apoptotic cells (±SD)</th>
<th>% Necrotic cells (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP in DMEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% PVP in 99% DMEM</td>
<td>5.7 (±0.5)</td>
<td>23.2 (±1.7)</td>
<td>64.5 (±8.5)</td>
</tr>
<tr>
<td>5% PVP in 95% DMEM</td>
<td>49.3 (±8.0)</td>
<td>21.0 (±0.7)</td>
<td>22.6 (±6.3)</td>
</tr>
<tr>
<td>10% PVP in 90% DMEM</td>
<td>69.7 (±8.2)</td>
<td>12.9 (±2.6)</td>
<td>11.6 (±5.7)</td>
</tr>
<tr>
<td>20% PVP in 80% DMEM</td>
<td>55.5 (±11.0)</td>
<td>9.4 (±2.3)</td>
<td>26.6 (±4.7)</td>
</tr>
<tr>
<td>40% PVP in 60% DMEM</td>
<td>4.6 (±1.3)</td>
<td>5.0 (±2.2)</td>
<td>75.0 (±6.8)</td>
</tr>
<tr>
<td>PVP and HS in DMEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% PVP in 85% DMEM and 10% HS</td>
<td>53.2 (±8.7)</td>
<td>17.6 (±2.6)</td>
<td>25.5 (±5.7)</td>
</tr>
<tr>
<td>10% PVP in 80% DMEM and 10% HS</td>
<td>72.1 (±8.1)</td>
<td>9.2 (±0.5)</td>
<td>11.1 (±5.2)</td>
</tr>
<tr>
<td>20% PVP in 70% DMEM and 10% HS</td>
<td>61.8 (±12.5)</td>
<td>10.8 (±4.2)</td>
<td>24.5 (±6.0)</td>
</tr>
<tr>
<td>PVP and FCS in DMEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% PVP in 85% DMEM and 10% FCS</td>
<td>55.4 (±11.0)</td>
<td>15.4 (±2.1)</td>
<td>19.3 (±2.6)</td>
</tr>
<tr>
<td>10% PVP in 80% DMEM and 10% FCS</td>
<td>69.2 (±6.7)</td>
<td>9.3 (±1.6)</td>
<td>15.0 (±2.3)</td>
</tr>
<tr>
<td>20% PVP in 70% DMEM and 10% FCS</td>
<td>64.3 (±6.4)</td>
<td>13.2 (±4.7)</td>
<td>19.6 (±2.3)</td>
</tr>
<tr>
<td>10% PVP in 50% DMEM and 40% FCS</td>
<td>70.5 (±3.1)</td>
<td>9.4 (±1.0)</td>
<td>6.3 (±4.0)</td>
</tr>
<tr>
<td>10% PVP in 10% DMEM and 80% FCS</td>
<td>72.5 (±2.9)</td>
<td>12.3 (±1.7)</td>
<td>6.6 (±4.0)</td>
</tr>
</tbody>
</table>

PVP, polyvinylpyrrolidone.
the media with PVP, we have repeated the cryopreservation experiments with 10% PVP as CPA in the presence of 40% and 80% FCS (Table 2). As seen in Table 2, increasing the percentage of FCS from 10% to 80% with 10% PVP did not significantly increase the percentage of viable cells or decrease the percentage of necrotic or apoptotic cells. These observations indicate that the presence of serum may not be vital to the postthaw viability and apoptotic, necrotic response of P1 ASCs frozen in PVP and DMEM (and in the absence of DMSO).

To address whether the presence of PVP and the absence of serum and DMSO during cryopreservation affects postthaw adipogenic and osteogenic differentiations in ASCs, we utilized toluidine blue, oil red O, and alizarin red to stain the undifferentiated ASCs, adipocytes, and osteoblasts, respectively. As described earlier, the staining was then extracted from the differentiated cells and the OD at the appropriate wavelength was determined. The P1 ASCs after 14 days of adipogenic induction and 21 days of osteogenic induction displayed morphological features consistent with adipogenesis or osteogenesis from control (unfrozen) cells (Fig. 3). Representative photomicrographs in Figure 3 show undifferentiated ASCs stained positive with toluidine blue (Fig. 3A–C), adipocytes stained positive with oil red O (Fig. 3D–F), and mineralized (osteoblast) cultures stained positive with alizarin red (Fig. 3G–I). Control experiments showed no significant morphological differences between the cells cryopreserved in the presence of either 80% HS with 10% DMSO in DMEM (Fig. 3A, D, G) and with FCS (Fig. 3B, E, H). The quantitative OD measurements from the eluted stains indicated a slight decrease in the differentiation potential of the ASCs when cryopreserved in the presence of 10% PVP in DMEM (and in the absence of FCS and DMSO) when compared with that obtained from cells frozen with 80% FCS and 10% DMSO in DMEM (control). Specifically, the OD of the extracted dye from the control cells was 0.63±0.05 and the percentage differentiation was assumed to be 100%. The OD of the extracted dye from the cryopreserved cells (10% PVP in DMEM) was 0.58±0.11 (osteogenesis) and 0.61±0.08 (adipogenesis). The percentage differentiation of the cryopreserved cells (10% PVP in DMEM) was ~85% (osteogenesis) and ~90% (adipogenesis). Thus, the decrease in the OD measurement (or the differentiation ability) between control cells and cryopreserved cells was statistically not significant (95% confidence level).

Next, we utilized RT-PCR to determine whether the cryopreservation of ASCs in the presence of PVP and in the absence of serum and DMSO affected the expression of genes involved in adipogenic differentiation. Serum-free cryopreservation in the presence of 10% PVP did not have a significant effect on the expression of adipogenic differentiation markers such as PPARγ2, adiponectin, and leptin when compared with ASCs cryopreserved in the control media (80% FCS and 10% DMSO in DMEM) (Fig. 4). In two independent analyses, the cells cultured under adipogenic conditions displayed an induction of the three mRNAs relative to control levels.

Discussion

Increasing the serum concentration in the cryopreservation medium has often been shown to increase the postthaw survival rate of cells that are difficult to preserve; serum concentrations as high as 90% have been reportedly used.32,39,40 Our results demonstrated that the choice of serum (either animal or human) did not alter the phenotype characteristics of frozen/thawed P1 ASCs. This valuable observation, if found to be valid for other cells as well, should allow researchers to shift to autologous serum rather than using xenogeneic serum which may contain anomalous and infectious particles in future cell cryopreservation experiments. However, the use of autologous serum does not completely eliminate the risk of bacterial toxins and microorganisms such as viruses, bacteria, and fungi.8 Autologous serum could be an alternative for ASC cryopreservation and culture. However, production of autologous serum is a painstaking and time-consuming process and the amount of autologous serum necessary for storage and sufficient expansion of ASCs would exceed the amount a patient could safely provide.9 Therefore, complete elimination of unhealthy serum products is the best alternative for cryopreservation storage of ASCs for future clinical applications.

The temperature/time history experienced by the ASCs during the freezing process as well as the associated CRs measured using a temperature data logger indicated that the ASCs experienced different CRs at different time points when frozen in a media consisting of DMEM and PVP when compared with media consisting of DMEM with DMSO and serum (as described earlier in the Results section). Additionally, the cells frozen with PVP experienced significant supercooling of the extracellular media. Briefly, on the basis
of measured osmolality, the phase change temperature of DMEM media containing 10% PVP is \(-0.6^\circ C\). However, in our freezing experiments the phase change of the extracellular medium in the presence of PVP occurred at \(-17^\circ C\), that is, with a significant supercooling. This supercooling might have resulted in the formation of damaging ice crystals and hence the observed lower postthaw viability of ASCs frozen with PVP and DMEM when compared with cells frozen with serum and DMSO in DMEM (see Tables 1 and 2). This possibly deleterious supercooling associated with the presence of PVP in the freezing media could not be eliminated even with the addition of ice nucleating agents such as *Pseudomonas syringae* (data not shown).

In our experiments, extremely low (1%) and extremely high (40%) concentrations of PVP caused significant reduction in the percentage of viable ASCs postfreeze/thaw. Obviously, this suggests that (i) a minimal percentage of PVP is needed before it can act as a cryoprotectant, and (ii) increasing the concentration of PVP beyond an optimum value causes toxic damage to the cells. The significant percentage of cells exhibiting necrosis when frozen with 1% PVP in DMEM may be explained by the fact that there was not enough cryoprotectant at freezing, leading to the formation of large and possibly damaging ice shards. In contrast, it is possible that the necrosis observed at 40% PVP in DMEM occurred before freezing, especially as there are reports that PVP is toxic in some systems.\(^{34,41}\) Our observations suggest that PVP is indeed toxic to ASCs at higher concentrations (>20%) and a possible remedial strategy might be to expose ASCs to 40% PVP in a stepwise manner, as is currently performed with embryo freezing.\(^{42}\) Obviously, it is possible that the stepwise addition of PVP to higher concentrations (i.e., 40%) might further enhance the postthaw cell viability of ASCs cryopreserved in PVP (and possibly even exceed those obtained in control medium, i.e., 80% serum and 10% DMSO in DMEM). This is an avenue for exploration in future studies.

There are several hypotheses reported in the literature concerning polymer cryoprotection but none are widely accepted. These hypotheses include the following: (i) the possibility that high-molecular-weight polymers such as PVP could act colligatively and protect cells during freezing\(^{10}\), (ii) the coating of cell membranes by PVP has been suggested as an alternative mechanism of polymer cryoprotection\(^{30,43}\), (iii) at high concentrations, PVP exerts considerable effects on freezing point depression of the system, in excess of that...
predicted from its molar concentrations.\textsuperscript{40,27} Thus, the protection offered by PVP is possibly linked to its nonideal behavior at high concentrations in aqueous solutions. Further work is clearly needed to elucidate the cryoprotective mechanism of PVP and to mechanistically (biophysically) develop and optimize cryopreservation media for ASCs as well as other cell types.

**Conclusion**

To the best of our knowledge, this is the first attempt to develop serum-free, DMSO-free solutions for cryopreservation of ASCs. Toward this goal, we have investigated the effects of a range of PVP concentrations on the postthaw behavior and differentiation ability of ASCs in the absence of any serum and DMSO. The results of this study demonstrate that the presence or absence of serum did not significantly alter the viability of ASCs frozen with 10% PVP. In two independent trials, cDNAs were prepared from the total RNA and were used in polymerase chain reactions together with primers corresponding to peroxisome proliferator-activated receptor \( \gamma \)2 (PPAR\( \gamma \)2) (\( A, B \)), adiponectin (\( C, D \)), and leptin (\( E, F \)). The cells cryopreserved in control media and media with 10% PVP expressed lineage-specific markers in the same manner. All experiments were performed in triplicate. Values are reported as the mean ± standard deviation. Color images available online at www.liebertonline.com/ten.

\( \text{FIG. 4.} \) Reverse transcription–polymerase chain reaction analysis of adipogenic specific markers expressed in induced and noninduced ASCs cryopreserved either in control medium (80% FCS + 10% DMSO in DMEM) or in serum-free medium containing 10% PVP. In two independent trials, cDNAs were prepared from the total RNA and were used in polymerase chain reactions together with primers corresponding to peroxisome proliferator-activated receptor \( \gamma \)2 (PPAR\( \gamma \)2) (\( A, B \)), adiponectin (\( C, D \)), and leptin (\( E, F \)). The cells cryopreserved in control media and media with 10% PVP expressed lineage-specific markers in the same manner. All experiments were performed in triplicate. Values are reported as the mean ± standard deviation. Color images available online at www.liebertonline.com/ten.
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Disclosure Statement

No competing financial interests exist.

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