International Journal of Heat and Mass Transfer 51 (2008) 5650–5654

Contents lists available at ScienceDirect

International Journal of Heat and Mass Transfer

journal homepage: www.elsevier.com/locate/ijhmt

The effect of two different freezing methods on the immediate post-thaw membrane integrity of adipose tissue derived stem cells

R. Fuller, R.V. Devireddy *

Bioengineering Laboratory, Department of Mechanical Engineering, Louisiana State University, Baton Rouge, LA, USA

ARTICLE INFO

Article history:
Received 2 November 2007
Received in revised form 4 April 2008
Available online 4 June 2008

Keywords:
Directional cooling stage
Cooling rate
Membrane integrity
Controlled rate freezer

ABSTRACT

The effect of directional cooling on the immediate post-thaw membrane integrity of adipose tissue derived adult stem cells (ASCs) was investigated using a directional solidification stage (DSS). Several passages, including Passage-0 (P0), Passage-1 (P1), and Passage-2 (P2), obtained from the suspended culture of stromal vascular fraction (SVF) of the ASCs were used for this study. ASCs from P0, P1, and P2 were cooled at either 1, 5, 20, or 40 °C/min to an end temperature of −80 °C in the presence and absence of a cryoprotective agent (dimethylsulfoxide, DMSO). After freezing to −80 °C, the samples were thawed at 200 °C/min and the ability of the frozen/thawed ASCs to exclude fluorescent dyes was assessed. ASCs frozen using the DSS in the absence of DMSO were found to have a lower post-thaw cell membrane integrity (confidence level of 95%), when compared with the ASCs frozen in the presence of 10% volume/volume (v/v) ratio of DMSO. Intriguingly, a comparison with corresponding data for ASCs that were frozen using a commercially available controlled rate freezer (CRF) suggests that the directionally cooled ASCs (both in the absence and presence of DMSO) exhibit a significantly lower post-thaw cell membrane integrity (confidence level of 95%). This lowering of post-thaw cell membrane integrity for ASCs frozen using the DSS is postulated to be related to the differences in the nature and the associated damaging effects of ice crystals formed in the DSS vs. the commercial freezer.

1. Introduction

Preservation of adult stem cells in the frozen state for later clinical and biotechnological use is an area of ongoing interest. Cryopreserved adipose-derived adult stem cells, ASCs (or progenitor cells isolated from fat tissue exhibiting multi-lineage potential and differentiation *in vitro* toward fat, cartilage, muscle, bone and nerve cells) could provide a steady supply of these cells to develop tissue engineering strategies and cell-based therapies [1–6]. To rationally develop optimal freezing storage protocols, it is instructive to perform quantitative freezing studies of isolated ASCs in the presence and absence of cryoprotective agents.

Development of optimal cryopreservation protocols necessitates a fundamental understanding of the freezing process, i.e., the nature of the interaction between ice and the biological system being frozen. To this end, cryomicroscopes have been developed by several investigators including Diller and Cravalho [7] and Cosman et al. [8]. These cryomicroscopes were capable of imposing controlled and uniform temperatures throughout the cell system being frozen and concurrently allowed the visualization of the freezing process by utilizing a light microscope. An alternative device termed as the directional solidification stage (DSS) was originally described for use with biological systems by Rubinsky and Ikeda [9]. The DSS simulates the freezing process experienced by samples that are experiencing spatially varying temperature profiles or the temperature is non-uniform throughout the sample. The DSSs have been utilized to describe the mechanical interaction between ice crystals and cells, in which cells are pushed and deformed by the ice crystals [10–14], and coupled with a freeze-substitution procedure, they have also been utilized to determine the relative volumes of water and cells in tissue sections during a freezing process [15].

In the present study, we compared post-thaw viability of ASCs under corresponding cooling conditions from a traditional controlled rate freezer (CRF), where the ice front advances simultaneously from all directions, with the post-thaw viability of ASCs frozen using a custom-built DSS. Another important aspect of the present work is to study the effect of a cryoprotective agent (dimethylsulfoxide, DMSO) on the post-thaw survival of ASCs frozen using a CRF or a DSS. These results should help to better elucidate the link between an advancing ice front and cell survival, in the presence and absence of DMSO.

2. Materials and methods

All the human protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review

* Corresponding author. Tel.: +1 225 578 5891; fax: +1 225 578 5924.
E-mail address: devireddy@me.lsu.edu (R.V. Devireddy).

0017-9310/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijheatmasstransfer.2008.04.021

Board. Subcutaneous adipose tissue liposuction aspirates were provided by plastic surgeons in Baton Rouge, LA. All procedures were conducted under aseptic conditions according to a modification of methods outlined elsewhere [1–6,16–18]. Tissue samples (100–200 ml) were washed 3–4 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, re-suspended, and the centrifugation step repeated. The supernatant was aspirated and the pellet resuspended in culture medium (DMEM high glucose, 10% fetal bovine serum, 100 units penicillin/ml, 100 mcg streptomycin/ml, and 25 mcg amphotericin/ml). The cell suspension was plated at a density equivalent to 0.156 ml of liposuction tissue per square cm of surface area, using a 35 ml volume of culture medium per T225 flask. Cells were cultured for 48 h in a 5% CO_2, humidified, 37 °C incubator. At that time, the adherent cells were rinsed once with prewarmed PBS and the cells fed with fresh culture medium every 2–3 days until they reached approximately 75–80% confluence in approximately 7–10 days. At this time, the medium was 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 culture medium, centrifuged for 5 min at 1200 rpm (300*g*), the pellet re-suspended in a volume of 10 ml of culture medium, and the cell count determined by trypan blue exclusion. These cells were identified as Passage-0 (P0). The cells were reseeded in T225 flasks at a density of 5×10^3 cells per square cm. The cells were maintained in culture and passaged as described through successive Passages-0 to -2 (P0–P2). Thus, it takes approximately three to four weeks of culture to harvest P2 cells from the original stem cells isolated from human adipose tissue. Note that there is extensive data suggesting that these adherent cells exhibit multiple lineages when culture *in vitro* [1–6,16,17]. A recent study by Guilak et al. [5] shows, with the aid of histological and biochemical analyses, that pools of human adipose tissue derived adult stem cells can exhibit multiple differentiated phenotypes under appropriate *in vitro* culture conditions (briefly, 52% of the adult stem cell clones differentiated into two or more lineages; with more clones expressing osteoblasts, chondrocytes and neuron-like cells than adipocytes). Thus, there is clear evidence that the adherent cells cultured from stromal vascular fraction exhibit "stem cell" behavior and are termed as human adipose tissue derived adult stem cells (ASCs), in the present study.

The directional solidification stage (DSS) built by Rubinsky and Ikeda [9] consisted of hot and cold temperature copper bases (set at T_h and T_c, respectively) separated by a distance (D_{gap}). Freezing was achieved by placing the sample on a glass slide and moving the glass slide from the hot base to the cold base at a known velocity (V). The cooling rate (B) experienced by the sample is then described as: $B = \frac{T_h - T_c}{D_{gap}} \cdot V$. Note that during the cooling process, the two ends of the glass side are separately in thermal equilibrium with each base. We have recently built a DSS based on the design of Rubinsky and Ikeda [9] and our laboratory [19]. For the DSS freezing experiments, ~100 μl of ASC suspension was placed in a grooved microslide and traversed between the precisely controlled copper blocks at a constant velocity. Note that the first copper block was held at +4 °C and the second at −80 °C. All of the glass microslides had a circular milled well, perpendicular to the direction of microslide travel, within which the cell suspension was placed. The well was 3.2 mm wide (in the direction of travel) and of one mm depth (into the microslide surface). The presence of the well reduced spreading of the suspension over the microslide surface and allowed for a reproducible recovery of ~30 μl of suspension after freezing and thawing when a 100 μl sample was loaded initially [13]. The thawing rate of ~200 °C/min was achieved by quickly bringing the bottom of the glass slide into contact with a warm copper block maintained at 50 °C.

In the present study, the DSS experiments with different passages of ASCs were carried out both in the absence and presence of DMSO. Six separate DSS experiments were conducted in the absence of DMSO and an additional six in the presence of dimethylsulfoxide (DMSO; 10%, v/v) for each passage of ASCs. For experiments in the presence of DMSO, the cell suspensions were gently mixed with 10% DMSO as a cryoprotective solution (CPA), i.e., a one-step addition of CPA. The samples were equilibrated in the cryoprotective solution for ~10 min before the DSS experiments were performed.

ASCs were also frozen using a programmable controlled rate freezing (CRF) machine (Planer Series Kryo 560-16, TS Scientific, Perkasie, PA) [18]. Briefly, the samples were loaded into a six-well cell culture cluster (Corning Incorporated, Corning, USA). For CPA experiments, before freezing, the cell suspensions were gently mixed with 10% DMSO as a cryoprotective solution (CPA). Consistent with the DSS experiments described earlier, the samples were equilibrated in the cryoprotective solution for ~10 min before the freezing experiments were performed. The cluster was then loaded into the cryo-machine (which was precooled to +4 °C) and kept for 1 min for equilibration. The samples were then cooled at either 1, 5, 20 or 40 °C/min to an end temperature of −80 °C. As the samples are placed within a cooling chamber into which vapor-phase liquid nitrogen is being "pumped into" it is quite reasonable to assume that the whole sample/cell suspension is experiencing an uniform temperature drop, i.e., an uniform cooling rate. As the CRF machine we used was limited to a maximum controlled (and uniform) thawing/warming rate of 40 °C/min, the thawing rate 200 °C/min was obtained by removing the six-well cluster from the CRF machine and quickly bringing the bottom of the cluster into contact with a warm water bath maintained at 37 °C, as described earlier by Thirumala et al. [18]. The accuracy of the CRF (and the thawing rate obtained by placing the cluster in a warm water bath) was also verified, as described earlier by Thirumala et al. [18]. The cooling rates imposed by the CRF was within 5% for cooling rates of ⩽10 °C/min and within 10% for the highest cooling rates of 40 °C/min [15]. Similarly, we found that the thawing rate obtained by placing the cluster in the warm water bath was within 5% of 200 °C/min [18].

To further analyze the statistical significance of the differences in the measured post-thaw viability data between the various experimental conditions (without CPAs, with DMSO, between DSS and CRF and between ASC passages) we performed one-way analysis of variance (ANOVA) using SIGMASTAT from SPSS (Chicago; www.spss.com/software/science) at the usual probability level of $P = 0.05$. The results from the ANOVA analysis are presented in the next section.

3. Results and discussion

After the freeze–thaw process, the viability of cell suspensions was measured using calcein AM and propidium iodide (PI) as previously described [13,20]. A 10 μl sample of cell suspension was removed from the microslide and incubated with 2 μmol/l calcein AM and 3 μmol/l PI for 15–30 min at 37 °C. After incubation, cells were scored as either live or dead under a fluorescent microscope. Viability measurements of cells taken directly from the stock suspension without freezing were determined before and after every freeze–thaw experiment as a control; all experimental viability values were normalized to the average control viability. Control viabilities were all greater than 95%.

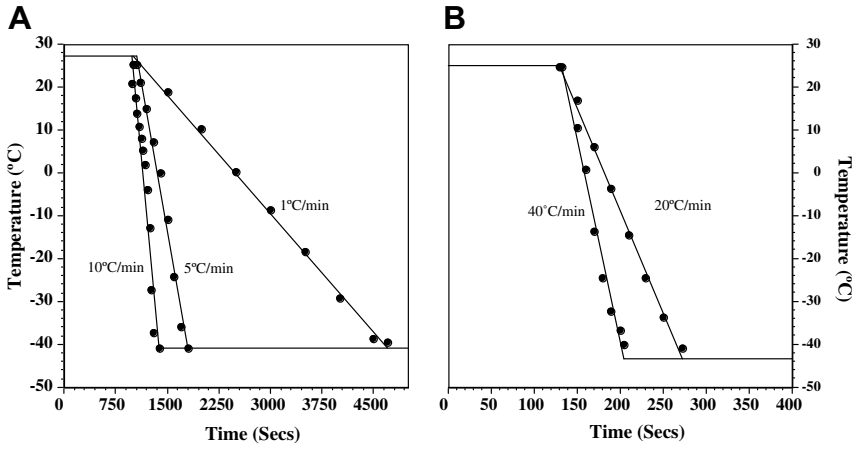

Fig. 1. Examples of measured thermal histories experienced during directional solidification of saline at 1, 5, and 10 °C/min (A) and at 20 and 40 °C/min (B) to end temperatures of −40 °C. In both the figures, temperature of the sample (°C) in the microslide groove is shown on the *Y*-axis while the time in seconds is shown on the *X*-axis.

By appropriately selecting the values for microslide velocity (V), gap distance (D_{gap}) and the temperatures of the copper blocks (T_h and T_c), we were able to achieve cooling rates that were within 10% of the required values, i.e., 1, 5, 10, 20 or 40 °C/min. As shown in Fig. 1A and B, the experimentally determined cooling rates for temperatures between 0 and −40 °C were ∼1.1, ∼5.2, ∼10.6

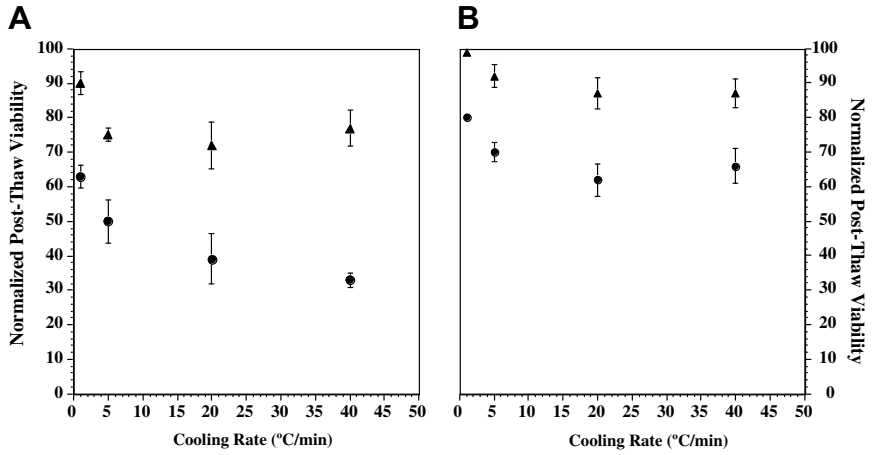

Fig. 2. Comparison of post-thaw viability of P0 ASCs frozen using the DSS (Fig. 1A) and the CRF (Fig. 1B). For each device, the post-thaw viability obtained in the absence (filled circles) and presence (filled triangles) of 10% (v/v) DMSO is shown. The error bars represent standard deviation in the data (n = 18). In both the figures, the immediate post-thaw ASC membrane integrity is shown on the *Y*-axis while the cooling rate imposed on the sample (°C/min) is shown on the *X*-axis.

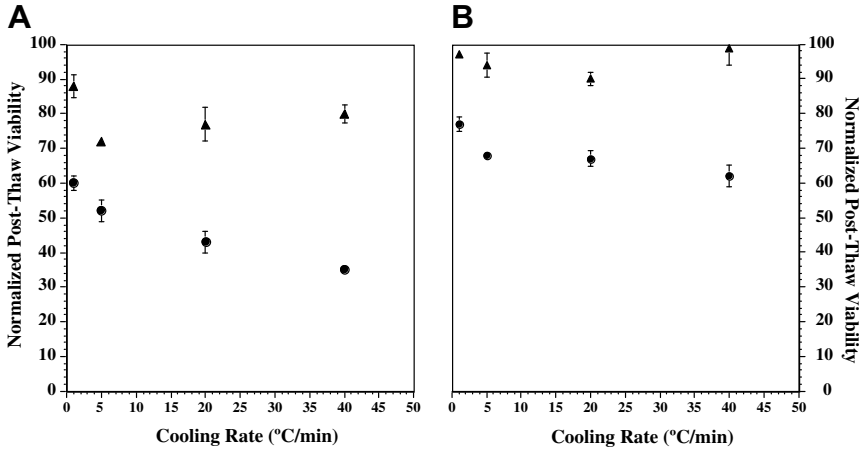

Fig. 3. Comparison of post-thaw viability of P1 ASCs frozen using the DSS (Fig. 1A) and the CRF (Fig. 1B). For each device, the post-thaw viability obtained in the absence (filled circles) and presence (filled triangles) of 10% (v/v) DMSO is shown. The error bars represent standard deviation in the data (n = 18). In both the figures, the immediate post-thaw ASC membrane integrity is shown on the *Y*-axis, while the cooling rate imposed on the sample (°C/min) is shown on the *X*-axis.

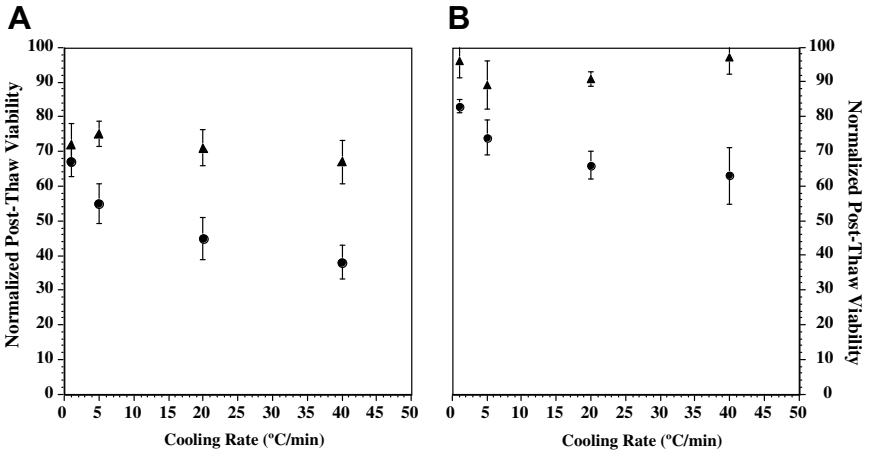

Fig. 4. Comparison of post-thaw viability of P2 ASCs frozen using the DSS (Fig. 1A) and the CRF (Fig. 1B). For each device, the post-thaw viability obtained in the absence (filled circles) and presence (filled triangles) of 10% (v/v) DMSO is shown. The error bars represent standard deviation in the data ($n = 18$). In both the figures, the immediate post-thaw ASC membrane integrity is shown on the *Y*-axis while the cooling rate imposed on the sample (°C/min) is shown on the *X*-axis.

(Fig. 1A) and ~21.7 and ~43.6 °C/min (Fig. 1B), respectively. These measurements were taken with a 5 mm gap spacing (Fig. 1A) and a 15 mm gap spacing (Fig. 1B) between the two copper platforms. The thermal histories shown in Fig. 1 were measured with a type-T thermocouple (Omega Inc., Stamford, CT, USA) with 0.5 mm bead diameter, immersed in 100 μl of saline in the microslide well. Data were collected using a HYDRA data acquisition system (Fluke Co., Everett, WA, USA.) at 2 s intervals [18].

A comparison of the P0, P1 and P2 ASCs post-freeze viability in the presence and absence of DMSO obtained using the DSS and the CRF is shown in Figs. 2–4, respectively. An examination of the data shows that the post-freeze viability of ASCs is significantly higher (confidence level > 95%), when they are frozen in the presence of DMSO, than in its absence. For example, in the absence of DMSO, the post-freeze viability of P0 ASCs decreases from a maximum of ~63% obtained at a cooling rate of 1 °C/min to a minimum of ~33%, when cooled at 40 °C/min. In contrast, the corresponding values obtained in the presence of DMSO for P0 ASCs are ~90% and ~77%, respectively. The data also suggests that the ASC post-freeze viability in the presence of DMSO remains fairly constant between the cooling rates investigated and intriguingly seems to exhibit a "shallow U" shape – this is in contrast to the "inverse U" shaped curve that is typically observed between cell viability and cooling rates for various cells [21,22].

A comparison of the post-freeze viability of P0, P1 and P2 ASCs frozen in the absence of DMSO using either the DSS or the CRF is shown in Table 1, while the corresponding data obtained in the presence of DMSO is shown in Table 2. Note that a cooling rate of 1 °C/min results in the highest post-thaw cell survival for both the devices. An examination of the data shows that the post-freeze viability of ASCs is uniformly higher (confidence level > 95%) when they are frozen using the CRF as opposed to using the DSS. This reduction in cell viability for the cells frozen using the DSS might be related to previously postulated mechanical/damaging interactions between the ice crystals and the cell membranes [10–14]. As time and resources become available, more detailed studies to elucidate these interactions as well as the changes in ice crystals formed when the solutions are frozen using the CRF and the DSS will be performed.

Table 1
Normalized average values (±standard deviations) of post-freeze/thaw membrane integrity of ASCs frozen in D-PBS

ASC passage	Cooling device	Cooling rate (°C/min)			
		1	5	20	40
P0	DSS	63 ± 3.4^{a}	50 ± 6.2^{a}	39 ± 7.3^{b}	33 ± 2.1^{b}
	CRF	80 ± 1.4^{c}	70 ± 2.9^{d}	62 ± 4.7^{d}	66 ± 5.1^{d}
P1	DSS	60 ± 2.2^{a}	52 ± 3.1^{a}	43 ± 3.0^{b}	35 ± 1.1^{b}
	CRF	77 ± 2.0^{c}	68 ± 1.0^{d}	67 ± 2.3^{d}	62 ± 3.1^{d}
P2	DSS	67 ± 4.1^{a}	55 ± 5.8^{a}	45 ± 6.1^{b}	38 ± 4.9^{b}
	CRF	83 ± 1.9^{c}	74 ± 5.2^{c}	66 ± 3.9^{d}	63 ± 8.1^{d}

The data for two different cooling devices is shown: DSS – directional solidification stage; and CRF – controlled rate freezer. For each passage, different alphabets in superscript correspond to statistically significant different data (95% confidence level), while the use of same alphabet signifies statistically not significant data (one-way ANOVA analysis).

Table 2
Normalized average values (±standard deviations) of post-freeze/thaw membrane integrity of ASCs frozen in D-PBS with 10% (v/v) DMSO

ASC passage	Cooling device	Cooling rate (°C/min)			
		1	5	20	40
P0	DSS	80 ± 3.4^{a}	75 ± 1.9^{b}	72 ± 6.7^{b}	77 ± 5.1^{b}
	CRF	89 ± 5.2^{d}	82 ± 3.2^{d}	77 ± 4.5^{d}	85 ± 2.3^{d}
P1	DSS	88 ± 3.2^{a}	72 ± 1.1^{b}	77 ± 5.0^{b}	80 ± 2.6^{b}
	CRF	97 ± 1.3^{d}	94 ± 3.5^{d}	90 ± 1.9^{d}	99 ± 4.9^{d}
P2	DSS	72 ± 6.1^{a}	75 ± 3.6^{a}	71 ± 5.2^{a}	67 ± 6.3^{a}
	CRF	96 ± 4.8^{b}	89 ± 6.9^{b}	91 ± 2.1^{b}	97 ± 4.7^{b}

The data for two different cooling devices is shown: DSS – directional solidification stage and CRF – controlled rate freezer. For each passage, different alphabets in superscript correspond to statistically significant different data (95% confidence level), while the use of same alphabet signifies statistically not significant data (one-way ANOVA analysis).

4. Conclusion

In conclusion, we have investigated the post-freeze thaw viability of P0, P1 and P2 ASCs frozen in the presence and absence of DMSO using a custom-built directional solidification stage (DSS). We have also investigated the effect of freezing P0, P1 and P2 ASCs using a commercially available controlled rate freezer (CRF). Our studies suggest that the post-freeze thaw viability of ASCs is significantly improved when there is DMSO in the freezing mixture. Additionally, under comparable cooling conditions, the post-thaw viability of cells frozen using the DSS are significantly lower than those obtained using a CRF. This lowering of post-thaw cell membrane integrity for ASCs frozen using the DSS is probably related to the different interactions between the solute/ice interface and the cell membranes.

Acknowledgments

The authors thank Dr. Elizabeth Clubb and Dr. James Wade at the Pennington Biomedical Research Center (PBRC) for supplying the liposuction aspirates and their many patients for consenting to participate in this protocol. Acknowledgements are also due to Dr. J. Gimble, Dr. S. Zvonic, Dr. E. Floyd, G. Kilroy and S. Thirumala at PBRC for processing the ASCs.

References

[1] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell 13 (2002) 4279–4295.
[2] J.M. Gimble, F. Guilak, Adipose-derived adult stem cells: isolation, characterization and differentiation potential, Cytotherapy 5 (2003) 362–369.
[3] K. Hicok, T. du Laney, Y.S. Zhou, Y.D.C. Halvorsen, C.D. Hitt, L.F. Cooper, J.M. Gimble, Human adipose-derived adult stem cells produce osteoid in vivo, Tissue Eng. 10 (2004) 371–380.
[4] B.M. Strem, K.C. Hicok, M. Zhu, I. Wulur, Z. Alfonso, R.E. Schreiber, J.K. Fraser, M.H. Hedrick, Multipotential differentiation of adipose tissue-derived stem cells, The Keio J. Med. 54 (2005) 132–141.
[5] F. Guilak, K.E. Lott, H.A. Awad, Q. Cao, K.C. Hicok, B. Fermor, J.M. Gimble, Clonal analysis of the differentiation potential of human adipose-derived adult stem cells, J. Cellular Physiol. 206 (2006) 229–237.
[6] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, Circ. Res. 100 (2007) 1249–1260.
[7] K.R. Diller, E.G. Cravalho, A cryomicroscope for the study of freezing and thawing processes in biological cells, Cryobiology 7 (1971) 191–199.
[8] M.D. Cosman, M. Toner, J. Kandel, E.G. Cravalho, An integrated cryomicroscopy system, Cryoletters 10 (1989) 17–38.
[9] B. Rubinsky, M. Ikeda, A cryomicroscope using directional solidification for the controlled freezing of biological material, Cryobiology 22 (1985) 55–68.
[10] C.H. Korber, M.W. Scheiwe, K. Wollhover, Solute polarization during planar freezing of aqueous salt solutions, Int. J. Heat Mass Transfer 26 (1983) 1241–1253.
[11] H. Ishiguro, B. Rubinsky, Mechanical interactions between ice crystals and red blood cells during directional solidification, Cryobiology 31 (1994) 483–500.
[12] H.R. Harmison, K.R. Diller, J.R. Walsh, C.M. Neils, J.J. Brand, Measurement of cell volume loss in the liquid region preceding an advancing phase change interface, Ann. New York Acad. Sci. 858 (1998) 276–283.
[13] D.J. Smith, W.M. Fahssi, D.J. Swanlund, J.C. Bischof, A parametric study of freezing injury in AT-1 rat prostate tumor cells, Cryobiology 39 (1999) 13–28.
[14] H. Takamatsu, B. Rubinsky, Viability of deformed cells, Cryobiology 39 (1999) 243–251.
[15] P.V. Pazhayannur, J.C. Bischof, Measurement and simulation of water transport during freezing in mammalian liver tissue, ASME J. Biomech. Eng. 119 (1997) 269–277.
[16] A.J. Katz, A. Tholpady, S.S. Tholpady, H. Shang, R.C. Ogle, Cell surface and transcriptional characterization of human adipose-derived adherent stromal (human adipose-derived adult stem) cells, Stem Cells 23 (2005) 412–413.
[17] A.M. Parker, A.J. Katz, Adipose-derived stem cells for the regeneration of damaged tissues, Expert Opin. Biol. Therap. 6 (2006) 567–578.
[18] S. Thirumala, S. Zvonic, E. Floyd, J.M. Gimble, R.V. Devireddy, The effect of various freezing parameters on the immediate post-thaw membrane integrity of adipose tissue-derived adult stem cells, Biotechnol. Prog. 21 (2005) 1511–1524.
[19] R. Fuller, R.V. Devireddy, Directional cooling of adult stem cells (Paper # IMECE2006-13490), IMECE ASME CD-ROM Publication, New York, NY, 2006.
[20] Y. He, Q. Dong, T.R. Tiersch, R.V. Devireddy, Variation in the membrane transport properties and predicted optimal rates of freezing for spermatozoa of diploid and tetraploid pacific oyster *Crassostrea gigas*, Biol. Reprod. 70 (2004) 1428–1437.
[21] P. Mazur, S.P. Leibo, E.H. Chu, A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells, Exp. Cell Res. 71 (1972) 345–355.
[22] M.A. Henry, E.E. Noiles, D. Gao, P. Mazur, J.K. Critser, Cryopreservation of human spermatozoa: IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function, Fertil. Steril. 60 (1993) 911–918.