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Clinical mesenchymal stem cell products experience functional changes in response to freezing

Kathryn Pollock¹, Darin Sumstad², Diane Kadidlo², David H McKenna², and Allison Hubel

¹University of Minnesota, Department of Biomedical Engineering

²University of Minnesota, Department of Clinical Cell Therapy

³University of Minnesota, Department of Mechanical Engineering

Abstract

Background—Current methods of MSC cryopreservation result in variable post thaw recovery and phenotypic changes due to freezing. The objective of this investigation is to determine the influence of *ex-vivo* cell expansion on phenotype of MSCs and the response of resulting phenotypes to freezing and thawing.

Methods—Human bone marrow aspirate was purchased from Lonza (Walkersville, MD). MSCs were isolated, and cells were assessed for total count, viability, apoptosis, and senescence over 6 passages (8–10 doublings/passage) in *ex vivo* culture. One half of cells harvested at each passage were re-plated for continued culture, and the other half were frozen at 1°C/min in a controlled rate freezer. Frozen samples were stored in liquid nitrogen, thawed, and reassessed for total cell count, viability, and senescence immediately and 48 hours post thaw.

Results—Viability did not differ significantly between samples pre freeze or post thaw. Senescence increased over time in pre freeze culture, and was significantly higher in one sample that experienced growth arrest both pre freeze and post thaw. Freezing resulted in similar initial post thaw recovery in all samples, but 48 hour post thaw growth arrest was observed in the sample with high senescence only.

Conclusion—High freeze senescence appears to correlate with poor post thaw function in MSC samples, but additional studies are necessary to obtain a sample size large enough to quantify results.

Keywords

apoptosis; freezing; function; mesenchymal stem cells; senescence; viability

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Corresponding author: Allison Hubel, PhD, University of Minnesota, 111 Church St. SE, Minneapolis, MN 55455, Phone: 612-626-4451, Fax: 612-625-4344, hubel001@umn.edu.

DISCLOSURE OF INTEREST

The authors have no conflicts of interest.

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INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are currently being investigated for a variety of clinical treatment applications. To date, over 300 clinical trials involve the use of MSCs, with over 2000 patients safely treated with MSCs.¹ MSCs are being investigated for the treatment of cardiovascular disorders (stroke, myocardial infarction), diabetes, connective tissue disorders (cartilage defects, osteonecrosis, limb ischemia), chronic obstructive pulmonary disease, nervous system disorders (multiple sclerosis, Parkinsons Disease, spinal cord injury), kidney diseases and more.^{2,3}

Effective preservation of MSCs is critical, in particular, for their use as a highly functional off-the-shelf therapy for patients. The ability to store the cells allows for completion of safety and quality control testing prior to use of the cells, permits transportation from the site of processing to the site of administration, and streamlines coordination of the cell therapy with patient care regimes.⁴ Development of MSC-based therapies requires standardization of methods of culture and cryopreservation. MSCs are typically cultured *ex vivo* and expanded to a sufficient cell number before patient administration. Uniform, optimized methods of cell expansion have not been developed, and media composition (basal media, serum and additional supplements), seeding density, expansion vessel and in vitro population doublings can vary considerably amongst investigators.

Ex vivo culture of cells has been associated with changes in cell phenotype.^{5,6} One such change observed in MSCs is the development of a senescent phenotype.⁷ Senescent cells exhibit an inflammatory secretome,⁸ and as such, may cause undesirable results in immunomodulatory therapies. *Ex vivo* culture of cells can also influence freezing response. Both hematopoietic progenitors and lymphocytes exhibited changes in subzero water transport and intracellular ice formation tendencies after *ex vivo* culture,^{9,10} which in turn can influence freezing response. Francois and colleagues quantified diminished response for indoleamine 2,3-dioxygenase (critical to immunomodulatory cell function) for frozen and thawed MSCs when compared to fresh non-frozen cells.¹¹ A recent study by Moll et al also showed that cryopreserved MSCs had reduced immunomodulatory and blood regulatory properties immediately post thaw.¹²

These temporal and freezing induced changes in cell behavior can lead to confounding outcomes for clinical studies using cryopreserved MSCs. One investigator hypothesizes that poor post thaw MSC function may have been responsible for the failure of a recent clinical trial.¹³ The objective of this investigation is to determine the influence of *ex vivo* cell expansion on phenotype of MSCs at harvest and the response of resulting phenotypes to freezing and thawing. This information will help clarify the influence of culture conditions on the biological characteristics of MSC products and potential shifts in composition or behavior resulting from the freezing process.

METHODS

CELL CULTURE AND PROCESSING

MSC culture and isolation—The MSCs used for this study were isolated from bone marrow purchased from Lonza (Walkersville, MD) and were shipped overnight on ice. Volume, cell count, and viability of samples were recorded upon arrival. Mononuclear cells (MNCs) were isolated from the bone marrow by Ficoll Paque Premium (GE Healthcare, Pittsburgh, PA) density gradient centrifugation and separation. Upon initial receipt, the 10mL bone marrow sample was diluted with 10mL of 0.9% saline. In a 50 mL conical tube, this dilute marrow cell suspension was carefully layered over 15mL of GE Ficoll Paque Premium. The resulting layered suspension was centrifuged at 300xg for 25 minutes at room temperature with no brake. The cell layer was collected, then washed with 50 mL of Hank's Balanced Salt Solution (HBSS – no phenol red, calcium, or magnesium, Lonza, Walkersville, MD) and centrifuged at 300xg for 5 minutes. A second wash was performed using the same procedure described above. The supernatant was discarded after both washes.

The MNCs isolated using this method were resuspended in mesenchymal stem cell complete culture medium (MSC CCM) composed of alpha-MEM base (Invitrogen, Grand Island, NY), 16.5% fetal bovine serum (FBS, Hyclone, Thermo Scientific, Waltham, MA), and 1% Glutamax (200mM, Invitrogen, Grand Island, NY). Characteristics of the cell population including cell count and viability were measured again at this stage, along with flow cytometry testing for the negative marker CD45, and positive marker CD90. Cells were seeded at a density of $1.0\text{--}1.5 \times 10^5/\text{cm}^2$ in appropriately sized tissue culture treated t-flasks (Corning, Corning, NY) at a media depth of 1.6mm.

Growth conditions and passaging—Cells were cultured in a 5% CO₂, 37°C incubator in MSC CCM on appropriately sized t-flasks. At 24 hours and 48 hours after seeding, non-adherent cells were removed via media change. In these culture conditions, only mesenchymal stem cells from the mononuclear cell population adhered to the surface. Cell enumeration and characteristics (doubling time, viability, senescence, and apoptosis) taken on and after the first harvest reflect only MSC characteristics. Media changes were performed every 2–4 days until cells reached the desired 70–80% confluence between days 8–12. When cells reached desired 70–80% confluence, they were passaged. MSCs were washed with Dulbeccos phosphate buffered saline (DBPS, Invitrogen, Grand Island, NY), removed from the culture surface with TrypLE select® (Invitrogen, Grand Island, NY), diluted with MSC CCM to quench the action of TrypLE select®, and centrifuged. Cells were resuspended in MSC CCM and analyzed for viability, senescence, and apoptosis. Half of the remaining cells that were not used for assays were seeded in new flasks at densities of 40–50 cells/cm², and the rest were frozen down at each passage. A total of 6 passages were performed, which corresponds to approximately 35–40 population doublings in healthy cells.

Freezing/Thawing—At each passage harvest, $1\text{--}10 \times 10^6$ cells were frozen down in a total volume of 1 mL per cryovial (Nunc/Nalge Thermo Scientific, Waltham, MA). Cells

were harvested, washed in DPBS, and resuspended in 5% Human serum albumin (HSA) to a concentration of $2\text{--}20 \times 10^6$ cells/ml (2x the desired freezing concentration). The cells were combined stepwise with an equal volume of 2X freezing media composed of 60% Plasmalyte A (Baxter, Deerfield, IL), 20% of 25% Human Serum Albumin (Baxter, Deerfield, IL), and 20% dimethylsulfoxide (DMSO, Bioniche Pharma, Belleville, Ontario, Canada). The final concentration of DMSO was 10% by volume. Cells were frozen in a controlled rate freezer with the following protocol:

1. Wait at 0.0°C (place sample in freezer at this step)
2. Wait at chamber = 0.0°C until sample = 1.0°C .
3. Ramp $-1^\circ\text{C}/\text{min}$ until sample = -12°C .
4. Ramp $-20^\circ\text{C}/\text{min}$ until chamber = -60°C .
5. Ramp $+15^\circ\text{C}/\text{min}$ until chamber = -18°C .
6. Ramp $-1^\circ\text{C}/\text{min}$ until sample = -60°C .
7. Ramp $-3^\circ\text{C}/\text{min}$ until sample = -100°C .
8. End.

After completion of the freezing protocol, cell vials were removed from the controlled rate freezer (using insulated gloves) and transferred quickly (<30 seconds) to liquid nitrogen storage to minimize transient warming of the sample. Samples were stored in liquid nitrogen for 30–45 days before being thawed for post thaw analysis.

Cells were removed from liquid nitrogen and thawed quickly in a 37°C water bath until just thawed/slushy. The cell solution was added dropwise to 10 mL of 37°C MSC CCM and centrifuged. The aspirate was discarded and cells were re-suspended in fresh MSC CCM. A portion of these cells was analyzed immediately for viability and senescence. Remaining cells were plated and analyzed for viability and senescence after 48 hours.

CELL CHARACTERIZATION ASSAYS

Acridine Orange/Propidium Iodide Viability Testing—Cells were tested for viability using acridine orange (AO, Life Technologies, Carlsbad, CA) and propidium iodide (PI, Life Technologies, Carlsbad, CA). A working solution of AO/PI was made by mixing 1.0 mL of 1mM AO stock in sterile water, 2.0mL of 0.5 mg/mL PI in DPBS, and 47mL of DPBS. The final working solution with concentrations of $20\mu\text{M}$ AO and $20\mu\text{g}/\text{mL}$ PI was combined with cells in a 1:15–1:20 dilution. At least 100 cells were counted in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA) under fluorescence. Under these conditions, viable live cells fluoresced green due to AO binding to nucleic acids via intercalation. Conversely, PI caused dead cells to fluoresce red, as it is membrane impermeant in healthy cells. Sample viability percentage was calculated by dividing the number of live cells by the number of total cells (live+dead) and multiplying by 100.

Senescence testing—Cells were tested for senescence using the Beta-glo® assay (Promega, Madison, WI) which measures lysosomal beta-galactosidase expression

associated with senescence in cells. In contrast to the conventional, absorbance-based Miller method, this assay is well suited for high throughput screening and has been used in this context for other organisms.^{14,15} Cells were centrifuged at 300xg for 5 minutes and resuspended with 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Life Technologies) in 0.9% NaCl. In white walled flat bottom 96 well plates (Corning), 100 µl of Beta-glo assay was added to 100µl of cell solution in each well. The plates were allowed to incubate for 30 minutes at room temperature, and were subsequently read for luminescence on a plate reader.

A control curve was generated to convert raw relative luminescence (RLU) to beta galactosidase percentage. A population of mesenchymal stem cells were forced into senescence by treatment with 100µM tert-butyl peroxide (t-BHP, Sigma, St. Louis, MO) 1 hour/day for 5 days. After each treatment, cells were washed with DPBS and fresh media was replaced. This senescent positive control population was seeded in a 96 well plate and serially diluted to produce a control curve. The linear best-fit line of this curve was used to calculate the percent beta-galactosidase expressed by experimental populations compared to fully senescent populations of the same size.

Flow cytometry—Flow cytometry was performed to assess viability, cell surface phenotype, and apoptosis of MSC samples. Viability was assessed using a standard 7-amino-actinomycin D (7-AAD, BD Biosciences, San Jose, CA) test. MSC samples were characterized for cell surface phenotype. Specifically, cells were stained for the presence of CD45 (BD Biosciences, San Jose, CA), and CD90 (BD Biosciences, San Jose, CA) as markers for cells surface phenotype. The acceptance criteria included that MSC were > 85% CD45⁻. MSCs should however be > 85% CD90⁺. Apoptosis was assessed using AnnexinV/PI staining. Cells entering the early stages of apoptosis stain positive for AnnexinV only, and cells that are undergoing necrosis or cell death stain positive for both AnnexinV and PI.

Karyotyping—G-banded karyotype analysis was performed on 20 metaphase cells to rule out presence of any numerical or structural chromosomal abnormality. If one cell tested abnormal, additional cells were analyzed to rule out the presence of a clonal abnormality. Documented benign heritable heteromorphisms, such as 9 small-p small-h, were not considered abnormalities. Pre-culture samples of marrow were available for comparison.

Statistics—Error bars represent standard deviations of triplicate measurements. A student's t-test was used to compare populations, and p-values reported are considered significant at $p < 0.05$. Triplicate beta-galactosidase measurements for senescence were compared between individual passages, while individual data points for passage viability, recovery, and apoptosis were pooled for all passages and compared between samples.

RESULTS

Characterization pre-culture

Each initial marrow sample from Lonza had high viability ($98\% \pm 2\%$). However, samples varied widely in initial concentration and total cell number. Initial samples had average

concentrations of $2.40 \times 10^7 \pm 1.28 \times 10^7$ cells/ml with total cell counts of $3.27 \times 10^8 \pm 1.85 \times 10^8$ (Table 1).

Initial samples underwent density gradient centrifugation to isolate mononuclear cells (MNCs). Total MNC counts averaged $1.24 \times 10^8 \pm 7.24 \times 10^7$ total cells. The MNC populations (Table 1) constituted similar proportions of their respective initial bone marrow population ($38\% \pm 4\%$), and had similar viability ($98\% \pm 1\%$). Senescence was low in the MNC population of all samples, with an average of $0.62\% \pm 0.63\%$. It is interesting to note that sample 2 had the lowest viability and total cell count before and after centrifugation and exhibited the highest senescence percentage.

Characterization of in vitro expansion cultures

Mesenchymal stem cells (MSCs) were isolated from the MNC population obtained after density gradient isolation via attachment to the culture surface. Flow cytometry performed on MSC populations confirmed appropriate expression for all markers in all samples at each passage (Table 2). Population doublings were set to zero for passage 1. Further population doublings were calculated based on cells seeded and harvested on each subsequent passage. Population doublings were consistent over time (as evidenced by a constant slope in Figure 1A) for samples 1 and 3. Both had relatively constant rates of doubling, undergoing approximately 0.7 population doublings per day for the duration of their culture through passage 6. Sample 2 exhibited slowing growth at passage 3, and population doublings plateaued in passages 4 and 5 at around 19.5, just over half of the total doublings experienced by samples 1 and 3. Passage 6 of sample 2 could not be performed due to sample growth arrest.

Viability for each sample remained high as culture time and population doublings increased when measured by both AO/PI and 7AAD (Table 2). Samples 1 and 3 exhibited viabilities of over 70% through 35+ population doublings, with a consistent gradual decrease in viability over time. Sample 2 exhibited a more rapid drop in viability as population doublings increased, with a final viability in passage 5 below threshold viability criteria for lot release when measured with the 7-AAD assay.

The beta-galactosidase percentage (used as a measure of total population senescence) tended to increase over time (Figure 1B). Other than the reading for sample 3 in P3 (20.8%), the beta-galactosidase percentages for samples 1 and 3 remained low (below 10%), and slowly increased over time after the first passage. Sample 2 displayed a trending increase in beta-galactosidase over time with final values in P4 of over 30%. Sample 2 values were also significantly higher ($p < 0.05$) than either of the other samples. Samples 1 and 3 did not have significantly different values ($p > 0.05$). Beta-galactosidase testing could not be performed on P5 of sample 2 due to low cell recovery at harvest.

Karyotyping performed on these initial samples showed no mutations in any passage of samples 2 and 3. Sample 1 presented with duplication on the long arm of chromosome 17 in 6% of cells in P5 and P6. Karyotyping could not be performed on P5 of sample 2 due to low cell recovery at harvest.

Apoptosis results for pre freeze cells were low, with <10% of cells testing positive for apoptosis in all passages of all samples except for P5 of sample 2 (Table 2). There was not compelling evidence of an increase in apoptotic percentage over time in culture for the number of samples tested.

Post thaw characterization

Total cell counts as well as viability, senescence, and apoptosis measurements were taken immediately upon thawing and after 48 hours in culture post thaw.

Immediate post thaw recovery was calculated by dividing the total number of viable cells post thaw by the total number of viable cells pre freeze in each frozen passage. Recovery was similar for all samples and passages (Figure 2A) with no obvious trending as a function of pre freeze population doublings. The majority of the data points fell between 80–100% recovery.

While there was no visible trending in the initial recovery of cells post thaw, after 48 hours of culture the population doublings experienced tended to decrease with increasing pre freeze passage number in all three samples (Figure 2B). Samples 1 and 3 experienced greater population doubling than sample 2, with a significant difference between samples 2 and 3 ($p<0.05$). Sample 2 experienced very little growth in its first three passages after thaw in addition to a decrease in cell number in P4 (as evidenced by the negative population doubling). In contrast, samples 1 and 3 showed almost a full population doubling in thawed samples from P1, which decreased as passage number increased. Sample 3 exhibited an increase in population doublings in P5 and P6.

Every passage of samples 1 and 3 (except sample 3 P3) experienced a significant ($p<0.05$) senescent enrichment post thaw when individual passages were compared pre freeze and post thaw (Figure 2C). Sample 2 experienced a significant decrease in its senescent fraction of cells in P1 and P3, but showed the same trends as pre freeze populations of increasing senescence with increasing population doublings. These senescent percentages dropped significantly ($p<0.05$) for each sample and passage at 48 hours post thaw compared to immediate post thaw percentages (Figure 2D).

DISCUSSION

The wide variation in cell behavior observed between the three separate samples characterized in this study is likely a result of sample quality differences and donor-to-donor variability^{16,17}. Variability upon initial receipt could result from differences in donor age, hemodilution of the sample, or variability in shipping time or temperature. The older age of the donor of sample 2 (41-yr old) could explain the initial lower cell counts and expression of higher levels of senescence in culture. In the future, quality control metrics such as total cell count, viability, and senescence measured on whole marrow could be used to screen samples upon initial receipt.

Flow cytometry performed on the MSCs confirmed that the cells used in the study expressed the cell surface phenotype consistent with MSCs (CD45 (<15%) and CD90 (>85%)) in all

passages of each sample. This appropriate expression indicates that MSCs were successfully isolated in this study, and the results presented are representative of MSC freezing behavior. Karyotyping abnormalities were low, with only 6% of cells in sample 1 exhibiting duplication in the long arm of chromosome 17 in passages 5 and 6. The limited chromosomal abnormality observed is consistent with literature.¹⁸ Due to the similar post thaw behavior observed in samples 1 and 3, the chromosomal abnormalities present do not appear to affect proliferative post thaw sample behavior.

Population doublings are a metric for assessing cellular health. Previous research by Bertolo et al has proposed using in vitro expansion scores for grading cells intended for transfusion.¹⁹ Cellular age has also been proposed as a metric for grading MSCs.²⁰ Growth kinetics for sample 2 were relatively normal initially (i.e., rates were the same as other samples for the first 2 passages) but even these early passages exhibited growth arrest post thaw. Samples that exhibit this type of growth arrest should not be utilized as transfusion products, but can go undetected using current selection criteria. This latent growth behavior may explain the poor performance of some cellular transfusion products *in vivo*. It is important to develop screening criteria to identify sub standard products like sample 2 before transfusion as growth arrest may not be observed or predicted before administration using current techniques.

Pre freeze viability remained high among all samples at all passages (well above the 70% viability selection criterion for infusion), and did not predict cellular vigor or proliferative potential. Pre freeze apoptosis also did not appear to be correlated with post thaw behavior. However, pre freeze senescence increased over time in all samples, and senescence beta-galactosidase values were significantly higher in sample 2 both pre freeze and immediately post thaw. Senescence may provide a valuable metric for sample screening in the future; even in early passages with normal population doublings and viability, sample 2 (which had poor post thaw cellular performance even at early passages) exhibited significantly higher senescence than other populations. We suggest future studies be performed to evaluate introducing pre freeze senescence as an additional lot selection criteria for MSCs intended for clinical use. Additional studies with a larger sample size are necessary to determine whether pre freeze senescence is statistically indicative of poor post thaw function, and if so establish a maximum acceptable senescence threshold for pre freeze populations.

In post thaw populations recovery was high for all cellular products, with most exhibiting 80–100+% recoveries independent of sample or passage number. However, when cells were cultured for 48 hours after thaw, differences in cellular population doublings became more apparent. If researchers or clinicians look solely at immediate post thaw recovery and viability, this potential for growth arrest may not be observed.

Senescence immediately post thaw was consistent with the trends seen in pre freeze populations. There was a significant enrichment of senescent cells in the post thaw populations of samples 1 and 3, and a significant decrease in several passages of sample 2.

Several groups have previously analyzed MSC response to cryopreservation. Bruder et al²¹ showed that cryopreserved MSC samples grown for extended passages in culture have

similar growth kinetics and ATPase activity to unfrozen cells. Mamidi et al²² showed that multiple rounds of cryopreservation did not functionally alter MSCs when compared to MSCs that had undergone cryopreservation only once. Dariolli et al²³ performed experiments to analyze porcine MSCs by measuring growth kinetics, senescence, and ability to respond to chemical cues. This previously published work as a whole suggests that cryopreservation does not alter the growth or differential behavior of cells.

However, freezing can induce changes in MSC phenotype and proliferation which has also been shown by Francois et al¹¹ and Moll et al¹². These studies show that high pre freeze MSC senescence appears to correlate with poor post thaw performance. In order to maximize the future success of clinical trials involving frozen MSC samples, it is imperative that future studies with larger sample size be performed to statistically evaluate the effects of freezing and storage on the regenerative and immunomodulatory properties of MSCs. A better understanding of cell behavior in response to freezing may improve lot selection criteria and enhance the effectiveness of transfused MSCs.

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ABBREVIATIONS

AO	Acridine Orange
CCM	Complete culture medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hank's balanced salt solution
HSA	Human serum albumin
MNC	Mononuclear cell
MSC	Mesenchymal stem cell
PI	Propidium Iodide
P_	Passage _
RLU	Relative luminescence unites

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Figure 1. Pre freeze MSC population doublings and beta galactosidase percentage

A – Population doublings over time. Samples 1 and 3 show robust growth, Sample 2 exhibits growth arrest beginning in passage 3. **B** – Beta-galactosidase percentage of expression indicative of senescence in population for MSCs in ex-vivo culture. Beta-galactosidase expression tended to increase over time in all samples. Sample 2 had significantly higher values ($p < 0.05$) at each passage than samples 1 and 3. Error bars represent standard deviations of triplicate beta-galactosidase measurements.

Figure 2. Post thaw MSC recovery and beta galactosidase characterization

A – Recovery of MSCs post thaw. Recovery = (viable cells pre freeze)/(viable cells post thaw)*100 and is used to account for cellular losses during freezing. Recovery does not vary significantly between samples and was consistently high (>80% for almost all samples) **B** – Population doublings experienced by thawed cells after 48 hours in culture. Sample 2 had low to no population doublings in every passage, with P4 exhibiting cellular losses (negative doublings). Growth in samples 1 and 3 was higher in early passage samples and declined with increasing passage number. **C** – Immediate pre freeze beta-galactosidase expression. Sample 2 had significantly higher initial post thaw expression than samples 1 and 3. Trends observed in post thaw samples matched those seen in pre freeze data, and were significantly higher in samples 1 and 3 ($p < 0.05$) when compared to pre freeze values. **D** – 48-hour post thaw beta-galactosidase expression. Expression values dropped significantly ($p < 0.05$) after 48 hours in culture for all samples when compared to immediate post thaw values.

Table 1

Patient data and initial mononuclear cell characterization

Sample	Patient age	Patient gender	Total isolated MNCs	Fraction of initial population	MNC viability (AO/PI)	MNC beta-galactosidase (senescence)
1	36	M	9.55 x 10 ⁷	34%	99%	0.00%
2	41	F	6.98 x 10 ⁷	41%	97%	1.27%
3	29	F	2.06 x 10 ⁸	39%	99%	0.60%
Average			1.24 x 10 ⁸	38%	98%	0.62%
Standard deviation			7.24 x 10 ⁷	4%	1%	0.63%

Table 2

Pre freeze MSC characterization at each passage

Sample	Passage	Surface marker characterization %		Viability %		Apoptosis % <i>Annexin V+/PI-</i>	Karyotype results
		<i>CD45</i>	<i>CD90</i>	<i>AO/PI</i>	<i>7-AAD</i>		
1	P1	1.80	99.55	99	93	9.54	Normal
	P2	0.33	99.76	99	90	3.73	Normal
	P3	0.12	99.85	99	83	2.00	Normal
	P4	0.21	99.41	98	77	6.54	Normal
	P5	0.55	99.14	97	80	5.03	Duplication of Chrm 17 in 6%
	P6	0.24	98.67	93	77	3.94	Duplication of Chrm 17 in 6%
2	P1	2.09	98.92	94	90	2.74	Normal
	P2	0.42	99.84	99	81	1.41	Normal
	P3	0.47	99.47	92	83	2.31	Normal
	P4	0.74	99.58	95	80	3.68	Normal
	P5	0.52	90.16	88	34	21.61	-
	P1	9.72	92.63	94	92	0.89	Normal
3	P2	0.19	99.88	98	86	2.71	Normal
	P3	0.22	99.81	97	91	1.61	Normal
	P4	1.31	99.95	96	91	1.55	Normal
	P5	0.22	99.67	96	85	5.67	Normal
	P6	0.24	97.35	93	93	2.83	Normal