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Effect of holding time, temperature and different parenteral solutions on viability and functionality of adult bone marrow-derived mesenchymal stem cells before transplantation

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Abstract

Mesenchymal stem cells (MSCs) have the ability to proliferate and differentiate into various lineages, given the appropriate microenvironment, thus making MSCs promising candidates for cell transplantation. For clinical applications, MSCs need to be stored in optimal conditions so that they may be transported and made available as an off-the-shelf product for companies to market. Freshly harvested and cultured or frozen–thawed bone marrow-derived MSCs were prepared for cell transplantation. Both freshly cultured or frozen–thawed MSCs were washed and resuspended in parenteral solutions, either 0.9% saline, Dulbecco's phosphate-buffered saline (DPBS), plasmalyte A or 5%dextrose and held for 2, 4, 6 and 8 h at 4 °C, 37 °C and RT (22 °C). The viability of the cells, differentiation capability and expression of cell surface markers were analysed. MSCs harvested from fresh cultures, resuspended in the parenteral solutions and maintained at 4 °C for 6 h showed more than 90% viability, and the viability was appreciably better when suspended in 5% dextrose at 4 °C for 8 h. In contrast, frozen–thawed cells can be held for a maximum of 2 h after thawing before losing their viability significantly below permissible limits for transplantation. We are reporting for the first time the effect of various parenteral solutions, holding times and temperatures on the viability and functionality of bone marrow-derived freshly cultured or frozen–thawed MSCs for transplantation. Our results suggested that freshly harvested MSCs can be held for 8 h at 4 °C in 5% dextrose or for up to 6 h at 4 °C in saline, DPBS or plasmalyte A. Freeze–thawed MSCs can be held for a maximum of 2 h in plasmalyte A before transplantation without affecting their viability and ability to differentiate. Copyright © 2008 John Wiley & Sons, Ltd.

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Keywords **cell viability; MSC; parenteral solutions; freeze–thaw; temperature; transplantation**

1. Introduction

Mesenchymal stem cells (MSCs) have an extensive capacity for self-renewal, proliferation and differentiation into several distinct mesenchymal lineages. Under appropriate conditions, MSCs are able to differentiate into multiple lineages, such as osteoblasts, adipocytes or chondrocytes (Pittenger *et al*., 1999). MSC have the potential to differentiate into other types of tissue such as cardiac, neural, hepatic, renal and muscle tissues. Animal studies have shown that the MSCs have the natural capacity to follow the cues released at the lesion site and home into the site of injury (Satake *et al*., 2004; Phinney and Isakova, 2005). Recently it has been hypothesized that the MSCs are also capable of releasing cytokines, which may mobilize the already existing stem cell niches that have the inherent potential to differentiate into particular

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lineages. All these properties collectively make MSCs a very promising cell transplantation candidate for certain incurable diseases, such as spinal cord injury, Parkinson's disease, motor neuron disease, multiple sclerosis, limb ischaemia, myocardial infarction. etc.

However, it is believed that these cells require a very strictly controlled environment in order to remain viable and healthy from the time of cell processing and while being transplanted into patients. Many surgical processes for stem cell transplantation take a long time, e.g. burr hole surgeries for Parkinson's disease or intra-coronary injections for myocardial infarction. It may likely happen that the viability of the MSCs may decrease while waiting for transplantation.

In order to use MSCs for stem cell therapy, it is therefore necessary to identify: (a) the most effective and safe approach for optimum injectable conditions under which they may be administered to the patient; and (b) the limits to which they may be stretched without compromising quality and viability.

In this study we analysed the effect of different parenteral solutions, temperatures and holding times on the viability and functionality of MSCs after being removed from the standard culture conditions for stem cell transplantation.

2. Materials and methods

2.1. Human bone marrow samples

This study was approved by the Institutional Committee for Stem Cell Research and Therapy (IC-SCRT) and the Institutional Ethics Committee (IEC). Bone marrow from five patients (30 ml/patient) in the age range 20–35 years was aspirated from the upper posterior of the iliac crest using a heparinized syringe, under general anaesthesia, in a general operating theatre, after obtaining informed consent from the patients. Before taking the bone marrow, the patients were screened for human immunodeficiency virus (HIV1), hepatitis B (HBV), hepatitis C (HCV) and cytomegalovirus (CMV) as a mandatory screening test.

All the experiments were replicated three times ($n = 3$), using five different human bone marrow samples in order to avoid sample-to-sample variation.

2.2. Isolation and culture of mesenchymal stem cells

Bone marrow processing and subsequent cultures were carried out in a current good manufacturing practice (cGMP)-compliant clean room. Briefly, bone marrow was diluted (1 : 1) with Dulbecco's modified Eagle's medium, low glucose (DMEM-LG; Gibco-Invitrogen), and centrifuged at 1800 r.p.m. for 10 min to remove the anti-coagulant. The supernatant was removed and the bone marrow was again diluted with DMEM-LG. The bone marrow mononuclear cells (MNCs) were separated by the Ficoll density gradient method (1.077 g/ml density) in 50 ml centrifuge tubes (Falcon, Becton-Dickinson). Bone marrow MNCs accumulated on the Ficoll–plasma interface were isolated and washed again with DMEM-LG. Isolated mononuclear cells were plated into three T-75 cm^2 culture flasks (Falcon, Becton-Dickinson) and cultured in DMEM-LG supplemented with 10% fetal bovine serum (HyClone), 200 mM glutamax (Gibco–Invitrogen) and incubated at 37 °C and 5% humidified CO_2. The non-adherent cells were removed after 48 h and fresh medium added. Subsequently the medium was replenished every 48 h. Upon confluency, the cells were harvested with 0.25% trypsin–EDTA (Gibco–Invitrogen) and re-plated in a single one-cell stack (Corning Life Sciences; cell stacks are large cell culture containers used for bio-production). For large-scale production, MSCs were plated onto one 10-cell stack (Corning Life Sciences).

2.3. Characterization of MSCs

At the end of the last passage, surface expression of CD73-PE and CD90-PE (BD Biosciences) were determined in culture-expanded MSCs. Flow cytometry was performed on a LSR-II instrument (Becton-Dickinson) and data were analysed using FACS Diva software.

2.4. Cell viability assay

Our experiments were based on a workable model of having a certified central processing unit for human mesenchymal stem cells and catering to the needs of the surrounding tertiary hospitals of the country. We anticipated several difficulties the clinicians might encounter while handling the samples for transplantation.

In the first experiment, fresh cultures were used and were harvested just prior to transplantation and suspended in the respective parenteral solutions. In the second experiment, MSCs were freeze–thawed and resuspended in a solution suitable for transplantation. This would be applicable for transportation of cells for transplantation to hospitals far away from the central processing unit. It also is dependent on the purpose of the transplantation/requirement of the clinicians, e.g. for intravenous injection, 50 ml of cell suspension is allowed, but for stereotaxic injections or spinal cord injections the volume cannot exceed 1 ml and the cells need to be suspended in saline.

Keeping these constraints and requirements in mind, this entire experiment was designed to provide all scientists in the field with the baseline data for providing cells and storing cells with maximum viability.

2.5. Experiment 1: viability of fresh cultured cells

The cells at passage 4 were harvested using 0.25% trypsin–EDTA (Gibco–Invitrogen), washed and resuspended at a concentration of 100×10^6/ml (100 million

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was chosen in the study as the maximum number of cells that need to be injected, and to establish whether there is any reduction in viability with such a high cell number when suspended in such a low volume) in 0.9% injectable saline, plasmalyte A (Baxter), 5% dextrose and Dulbecco's phosphate-buffered saline (DPBS; Gibco–Invitrogen) for 2, 4, 6 and 8 h at 4 °C, 37 °C and room temperature (RT; 22 °C).

At the end of respective time points, the cells were incubated with 7-amino-actinomycin D (7-AAD), which intercalates into double-stranded nucleic acids and is excluded by viable cells but can penetrate the cell membranes of dying or dead cells, hence it was used for quantifying the viability of the cells, for 20 min in the dark. The cell viability was assessed by flow cytometry (LSR-II, Becton-Dickinson) using FACS Diva software. At the end of the last time point the surface expressions of CD73-PE and CD90-PE were also determined and compared with those of freshly harvested cells.

2.6. Experiment 2: viability of MSCs following freeze–thaw

Cells from the 10-cell stack at passage 4 were dissociated using 0.25% trypsin–EDTA and suspended in DMEM-LG supplemented with 10% fetal bovine serum (FBS). The MSCs were centrifuged at 1800 r.p.m. for 10 min and the supernatant was discarded. The cells were washed once with DMEM-LG supplemented with 10% FBS, then resuspended in freezing solution containing plasmalyte A supplemented with 5% human serum albumin (HSA; Baxter) and 10% dimethyl sulphoxide (DMSO; Sigma–Aldrich) at a final concentration of 100×10^6 cells/15 ml freezing solution. Cells were frozen in cryocyte bags (Baxter) using a programmable slow freezing unit (Planar Kryo 560-16). After freezing, the cells were stored in a liquid nitrogen vapour-phased cryocontainer (Chart Industries) for 30 days.

Cryocyte bags containing MSCs were removed from the vapour phase of the liquid nitrogen cryocontainer and placed in a 37 °C water bath. Thawed MSCs were centrifuged at 1800 r.p.m. for 10 min in order to remove cryoprotectant. The thawed cells were again resuspended in 0.9% injectable saline, plasmalyte A, 5% dextrose and DPBS for 2, 4, 6 and 8 h at 4 °C, 37 °C and RT. At the end of respective time points, the cells were incubated with 7-AAD for 20 min in the dark. The cell viability was assessed by flow cytometry, using FACS Diva software. The cells were also characterized for surface expression for CD73-PE and CD90-PE and compared with freshly harvested MSCs.

2.7. Experiment 3: viability of frozen–thawed MSCs without removing cryoprotectant

Cells from the 10-cell stack at passage 4 were dissociated using 0.25% trypsin–EDTA and suspended in DMEM-LG supplemented with 10% FBS. The MSCs were centrifuged at 1800 r.p.m. for 5 min and the supernatant was discarded. The cells were washed once with DMEM-LG supplemented with 10% FBS and resuspended in freezing solution containing plasmalyte A supplemented with 5% HSA and 10% DMSO at a total concentration of 100×10^6 cells/15 ml freezing solution. The cells were frozen using a programmable freezer (Planar Kryo 560-16) in cryocyte bags (Baxter) by slow freezing. After freezing, the cells were stored in a liquid nitrogen vapour-phased cryocontainer (Chart Industries) for 30 days.

The MSCs frozen in the 50 ml bags were thawed in a 37 °C sterile water bath and diluted to 50 ml by adding 35 ml plasmalyte A, so as to make a total volume of 50 ml. The cells were incubated for 2, 4, 6 and 8 h at 4 °C, 37 °C and RT. At the end of respective time points, the cells were incubated with 7-AAD for 20 min in the dark. The cell viability was assessed by flow cytometry using FACS Diva software and also characterized for surface expression for CD73-PE and CD90-PE and compared with freshly harvested MSCs.

2.8. Differentiation

At the end of last time point, i.e. 8 h for fresh cultured cells and 2 h for frozen–thawed cells, the MSCs showed maximum viability and were also checked for their ability to differentiate into adipocytes and osteocytes, as previously described (Phinney and Isakova, 2005).

Briefly, osteoblast differentiation was induced by culturing human MSCs in DMEM-LG supplemented with 10% FBS, 200 mM Glutamax (Invitrogen), 10^{-8} mM dexamethasone (Sigma-Aldrich), 30 µg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich) for 3 weeks. Fresh medium was replenished every 3 days. Calcium accumulation was assessed by Von Kossa staining. The differentiated cells were washed with PBS and fixed with 10% formalin for 30 min. The fixed cells were incubated with 5% $AgNO_3$ for 60 min under UV light and then treated with 2.5% sodium thiosulphate for 5 min. Images were captured using a Nikon Eclipse 90i microscope (Nikon, Japan; www.nikon.com) and Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA; www.mediacy.com).

To induce adipogenic differentiation, human MSCs were cultured for up to 3 weeks in DMEM-LG supplemented with 10% FBS, 200 mM Glutamax, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 µg/ml insulin and 100 µM indomethacin (all Sigma-Aldrich). Medium with inducing factors was replenished every 3 days. The cells were fixed in 10% formalin for 20 min. 200 µl oil red O staining solution was added and incubated for 10 min at room temperature, then the cells were rinsed five times with distilled water. The dye retained by the cells was eluted by incubation with 750 µl isopropanol (Merck) and images were captured using the Nikon Eclipse 90i microscope and Image-Pro Express software.

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2.9. Statistical analysis

The results were analysed using Student's *t*-test and statistical significance was accepted as $p < 0.05$. The results were expressed as mean ± standard deviation (SD) of the mean.

3. Results

Stem cells require a strictly controlled environment in order to remain viable and healthy from the time of cell processing and while being transplanted into patients. In this study we analysed the effect of different parenteral solutions, temperatures and durations on the viability and stability of bone marrow-derived MSCs after being removed from the standard culture conditions or freeze–thawed for stem cell transplantation.

In this study three different experiments were carried out. First, freshly cultured MSCs, harvested for transplantation, were resuspended either in (a) injectable saline, (b) plasmalyte A, (c) 5% dextrose or DPBS. In the second experiment, frozen MSC were thawed, washed and resuspended in either (a) injectable saline, (b) plasmalyte A or (c) 5% dextrose or DPBS; and in the third experiment frozen–thawed MSCs were directly diluted with plasmalyte A without removing the cryoprotectant. In all three experiments, the MSCs after resuspension were held for 2, 4, 6 and 8 h at 4 °C, 37 °C and RT. This was followed by analysis of viability, expression of cell surface markers and differentiation capability.

3.1. Experiment 1: viability of fresh cultured cells

Freshly cultured MSCs resuspended in injectable saline, 5% dextrose, plasmalyte A and DPBS at 4 °C after 2, 4 and 6 h of holding maintained more than 90% viability. The viability was gradually decreased after 8 h in saline, DPBS ($p < 0.05$) and plasmalyte A. However, the MSCs in 5% dextrose at 4 °C after 8 h maintained more than $90.65 \pm 3.03\%$ viability. The decrease was most apparent for the MSCs held at 37 °C in all the parenteral solutions (Figure 1). Similarly, at RT viability was significantly reduced in all the parenteral solutions except 5% dextrose, where viability was maintained up to almost 90% (89.2 ± 5.5), even after 8 h of holding.

MSCs that showed >90% viability at the end of experiment were analysed for surface expression markers, such as CD73-PE and CD90-PE, analysed by flow cytometry and compared with MSCs without storage. MSCs held at 5% dextrose for 8 h and MSCs without storage showed similar patterns of expression (Figure 2). MSCs resuspended in saline, plasmalyte A and DPBS for 2, 4, and 6 h at 4 °C and RT also showed similar expression patterns (data not shown). These results showed that the cell surface expression did not change after holding the MSCs in parenteral solutions at different temperatures, even after 8 h.

It is therefore concluded from this experiment that injectable saline or dextrose may be an appropriate resuspension solution for fresh cultured MSCs at 4 °C and can be used within 6 h for transplantation in the case of saline and 8 h in the case of dextrose. The MSCs can also be held in dextrose at RT for a maximum of 6 h before transplantation without losing their viability.

3.2. Experiment 2: viability of MSCs following freeze–thaw

The purpose of using frozen–thawed MSCs is to make MSCs available for critical cases and make the product available off the shelf, without forfeiting its viability and quality. In the present experiment, frozen MSCs were thawed and centrifuged for removal of cryoprotectant and resuspended in injectable saline, plasmalyte A, 5% dextrose and DPBS.

The viability of frozen–thawed MSCs resuspended in plasmalyte A and 5% dextrose at 4 °C or RT for 2 h was almost 90% as compared to that of injectable saline and DPBS. The cell viability was then decreased to $88.13 \pm 1.49\%$ after 4 h, when the cells were suspended in plasmalyte A at 4 °C, whereas in the other parenteral solutions viability was significantly decreased after 4 h and reduced to 30% after 8 h at all temperatures. Surprisingly, the initial viability of frozen–thawed MSCs in injectable saline after 2 h at 4 °C was 80% and continued to be maintained until 6 h, before it significantly dropped to 30% after 8 h.

Viability was significantly lower in saline and DPBS and gradually decreased over time when the cells were held at RT. The decrease was more apparent for MSCs held at 37 °C at all the time points and in all the solutions, hence this may not be a method of choice for resuspension. Therefore, the results demonstrated that plasmalyte A can be a parenteral solution of choice for frozen–thawed MSCs but should be used for transplantation within 2 h at 4 °C or RT (Figure 3).

Frozen–thawed MSCs stored in plasmalyte A were analysed for surface expression markers such as CD73-PE and CD90-PE, analysed by flow cytometry and compared with fresh MSCs. MSCs stored in plasmalyte A (Figure 4A) and 5% dextrose (Figure 4B) after 2 h of holding and MSCs without storage showed a similar pattern of expression. These results showed that the cell surface expression did not change after holding frozen–thawed MSCs for 2 h in plasmalyte A or 5% dextrose.

3.3. Experiment 3: viability of frozen–thawed MSCs without removing cryoprotectant

From the previous experiment, we inferred that plasmalyte A was the best choice for preparing the cell suspensions. These cells were frozen in plasmalyte A along with DMSO/cryoprotectant. Therefore, in this experiment the viability and stability of frozen–thawed MSCs diluted

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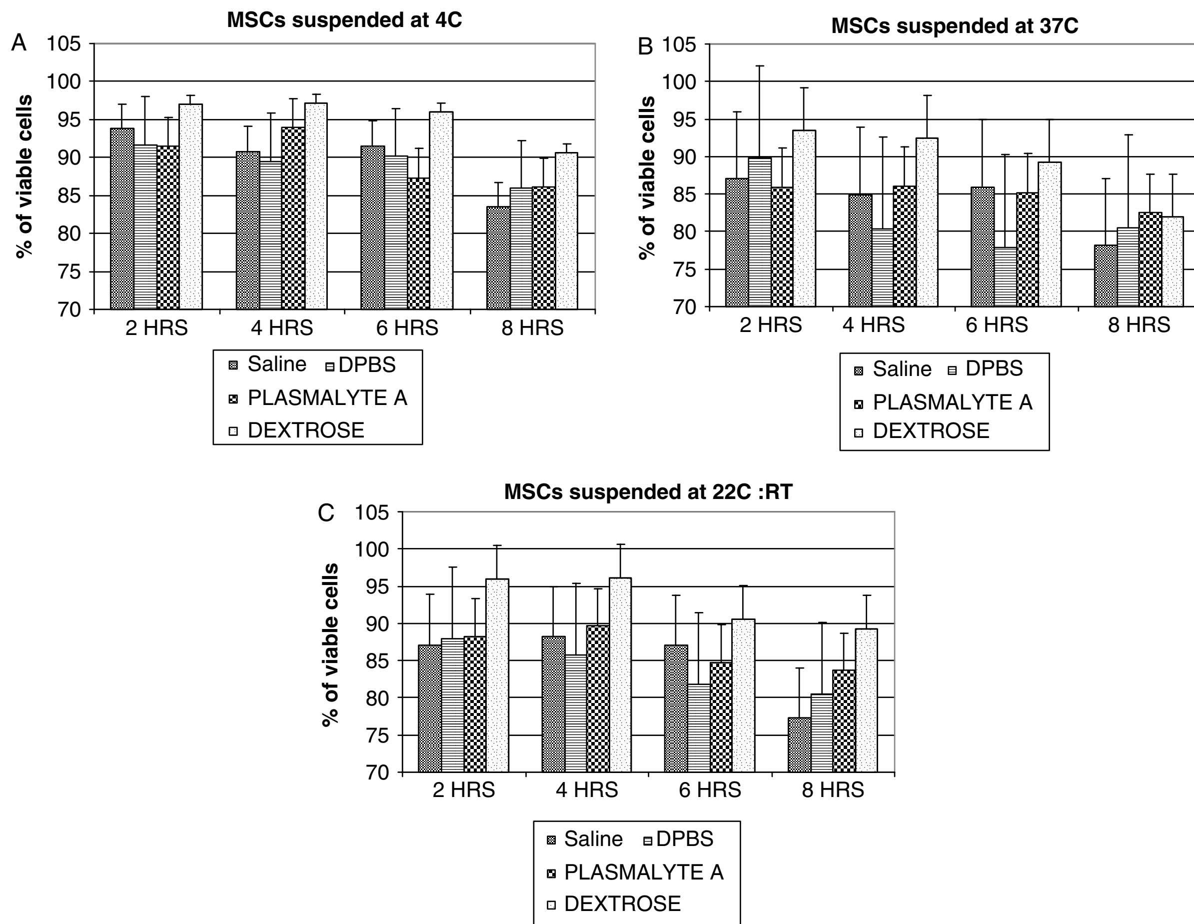

Figure 1. Adult bone marrow-derived mesenchymal stem cells (BM-MSCs) suspended in different solutions at different temperatures, harvested from fresh cultures. (A) Percentage of cell viability of freshly cultured (condition 1) adult BM-MSCs suspended in saline, DPBS, plasmalyte A and 5% dextrose at 4 °C at 2, 4, 6 and 8 h. The graph shows that the cell viability reduces from >90% to <90% when MSCs are suspended in saline, DPBS and plasmalyte A over time (2–8 h). The cells when suspended in 5% dextrose maintain cell viability >90% at 8 h, making it the solution of choice under these conditions. (B) Percentage of cell viability of condition 1 BM-MSCs suspended in the same solutions and times as for (A) but at 37 °C, clearly demonstrating that the viability of BM-MSCs is significantly <90% when maintained at this temparature; hence, MSCs should not be stored at 37 °C before transplantation. (C) Percentage of cell viability of condition 1 BM-MSCs suspended the same solutions and times as for (A) but at room temperature (RT), showing that the cell viability of BM-MSCs is significantly <90% at RT when suspended in saline, DPBS and plasmalyte A; when suspended in 5% dextrose the cell viability is maintained at 89.2 ± 5.5% after 8 h of holding. These results suggest that the MSCs can be held at room temperature for 8 h suspended in 5% dextrose prior to transplantation

with plasmalyte A without removing the cryoprotectant was evaluated. Frozen MSCs were thawed and resuspended by adding 35 ml plasmalyte A, so as to make a total volume of 50 ml and reduce the concentration of DMSO to a recommended dose for parenteral use. After dilution, the MSCs were kept for 2, 4, 6 and 8 h at 4 °C, 37 °C and RT.

The viability of the frozen–thawed MSCs was 95.4% at 4 °C after 2 h but reduced significantly after 4 h to 48.6%. Similarly, the viability of the frozen–thawed MSCs was 82.4% at RT after 2 h but reduced significantly after 4 h to 48.7%. Therefore, it is recommended that frozen–thawed MSCs should be used within 2 h of thawing and dilution.

MSCs resuspended in plasmalyte A were analysed for surface expression of CD73-PE and CD90-PE. We did not find any significant changes in surface expression between freshly cultured MSCs and in frozen–thawed MSCs held for 2 h in plasmalyte A (Figure 5).

3.4. Differentiation

MSCs that showed maximum viability at the ends of the experiments were subjected to *in vitro* differentiation into osteocytes and adipocytes. There was no change in the differentiation capability of cells after 8 h of holding, compared to 0 and 2 h following freeze–thaw when compared with 0 h (Figure 6).

4. Discussion

Mesenchymal stem cells (MSCs) are multipotent stem cells defined by their multilineage potential. They have the ability to self-renew, proliferate and differentiate into a variety of cell types. *In vitro*, after specific induction, MSCs have the potential to differentiate into adipocytes, osteocytes, chondrocytes, neurons, endothelial cells and

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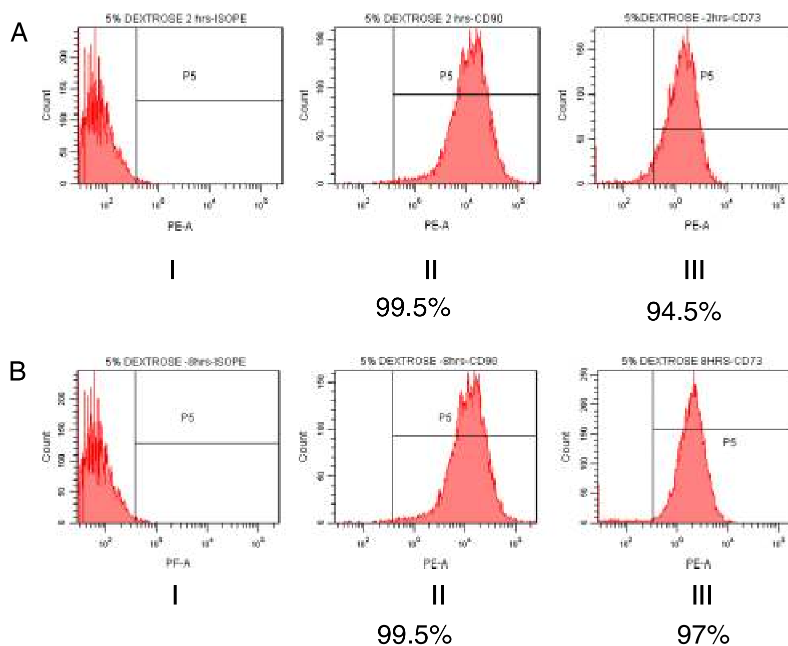

Figure 2. The MSCs that showed more than 90% viability at the end of experiment were analysed for surface expression markers such as CD73-PE and CD90-PE; analysed by a flow cytometer and compared with MSCs without storage. The figure shows the immunophenotyping of MSCs surface markers at 2 and 8 h, suspended in 5% dextrose harvested from fresh cultures. (A) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 2 h. II, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 99.5% positive for CD90. III, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 94.5% positive for CD73. (B) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 8 h. II, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 8 h; the cells are 99.5% positive for CD90. III, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 8 h; the cells are 97.0% positive for CD73

hepatocytes (Pittenger *et al*., 1999; Wakitani *et al*., 1995; Prockop, 1997; Sanchez-Ramos *et al*., 2000; Toma *et al*., 2001; Jiang *et al*., 2002; Kabos *et al*., 2002; Reyes *et al*., 2002; Schwartz *et al*., 2002). This differentiation potential makes MSCs a candidate for cell-based therapy for a variety of degenerative diseases (Sotiropoulou *et al*., 2006). Several clinical trials are currently being carried out, such as osteogenesis imperfecta (Horwitz *et al*., 2002) metabolic diseases (Koc *et al*., 2002), amyotrophic lateral sclerosis (Mazzini *et al*., 2003), myocardial infarction and graft-vs.-host disease (GVHD; Le Blanc *et al*., 2004), using MSCs.

In order to develop effective clinical-scale production of vast numbers of MSCs for cellular therapy in transplantation, immunotherapy or regenerative medicine, stringent quality control is required and it is mandatory to process them in a cGMP-compliant clean room. Building and maintaining clean rooms is expensive and difficult, especially in a hospital set-up. An ideal business model would be to develop a central processing laboratory for processing and up-scaling MSCs and transporting them to the hospitals through a distribution network. However, this escalates the requirement to transport, hold and store MSCs according to the patient's requirements at the hospital. For this, it is essential to transport, hold and store MSCs in optimal conditions so as to maintain their viability and multipotentiality for specified durations. However, there are no guideline for storing MSCs in suspension before transplantation. Limited studies have been carried out in order to determine optimal conditions for storing either freshly harvested MSCs or frozen–thawed MSCs for transplantation (Muraki *et al*., 2006; Kotobuki *et al*., 2005).

In the present study we focused on understanding the viability of MSCs suspended in different parenteral solutions and stored at different temperatures for different durations. We also examined the cell surface markers and their differentiation potential after holding for various durations.

Our data demonstrated that the viability of freshly harvested MSCs in all the parenteral solutions examined, 0.9% injectable saline, plasmalyte A and 5% dextrose, stored at 4 °C, maintain more than 90% viability until 6 h of storage. However, viability was found to be better when the MSCs were suspended in 5% dextrose at 4 °C until 8 h of holding, whereas the viability of MSCs stored at 37 °C and RT was lower and decreased significantly over time. Similar results were also observed by Muraki *et al*. (2006) and showed that decrease is most apparent for MSCs stored at 37 °C. This study showed that there was no difference of MSCs viability in PBS, saline and α-MEM. However, in this study the media used to store the cells may provide ample nourishment for the cells to remain viable and hence could be stored for 24 h at 4 °C. Phosphate-buffered saline (PBS) and α-MEM are only for *in vitro* use; they are not injectable solutions. These solutions are used in the laboratory for *in vitro* cultures only and are not suitable for clinical use.

In our study, we have used injectable solutions (saline, 5% dextrose, plasmalyte A) instead of α-MEM and PBS

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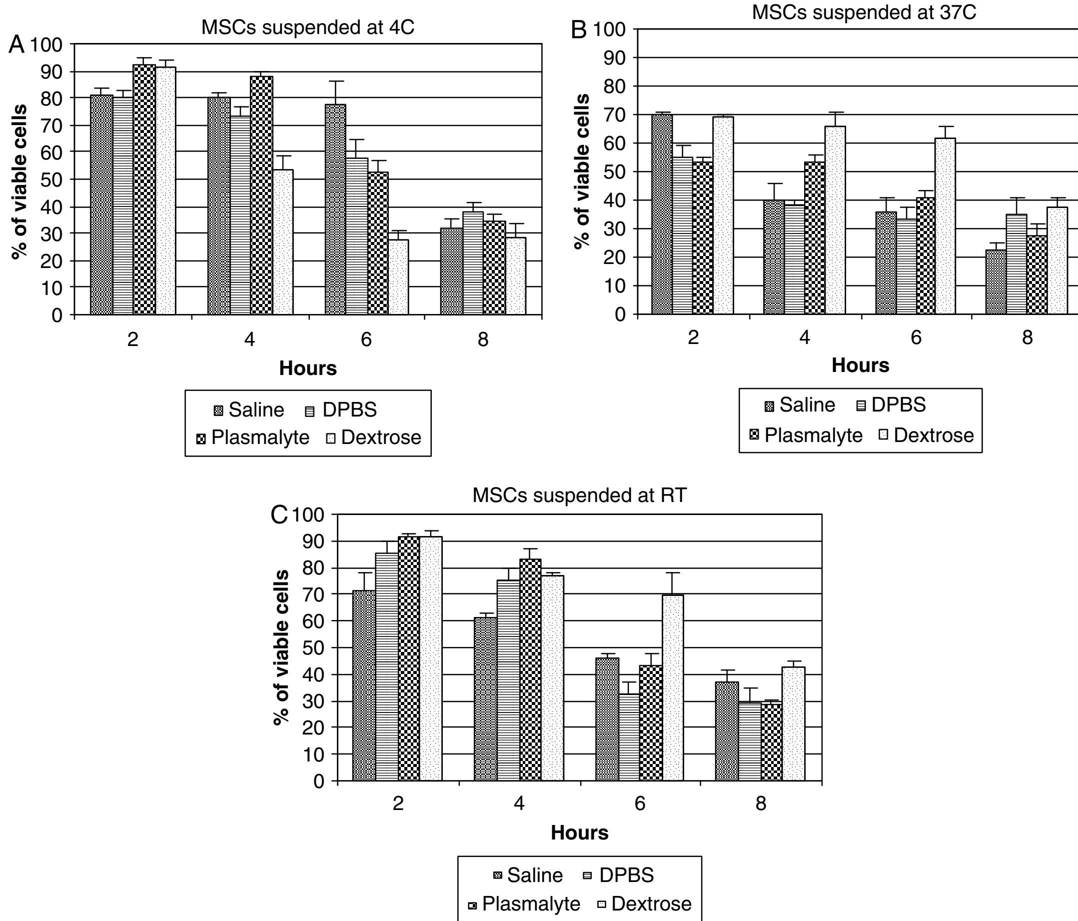

Figure 3. Adult BM-MSCs suspended in different solutions at different temperatures post-thawing, after removal of cryoprotectant. (A) Percentage of cell viability post-thawing (after removal of the cryoprotectant; condition 2) of adult BM-MSCs suspended in saline, DPBS, plasmalyte A and 5% dextrose at 4 °C at 2, 4, 6 and 8 h. (B) Percentage of cell viability of condition 2 BM-MSCs suspended in the same solutions and times but at 37 °C. (C) Percentage of cell viability of condition 2 BM-MSCs suspended in the same solutions and times but at room temperature (RT)

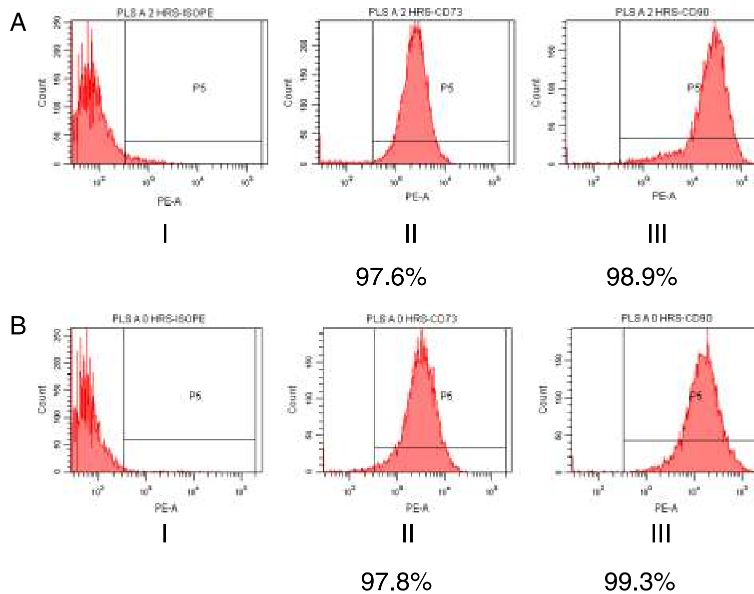

Figure 4. Immunophenotyping of MSCs surface markers at 0 and 2 h suspended in plasmalyte A following the freeze–thaw cycle. (A) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 0 h. II, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 0 h; the cells are 97.6% positive for CD90. III, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 0 h; the cells are 98.9% positive for CD73. (B) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 2 h. II, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 97.8% positive for CD90. III, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 99.3% positive for CD73

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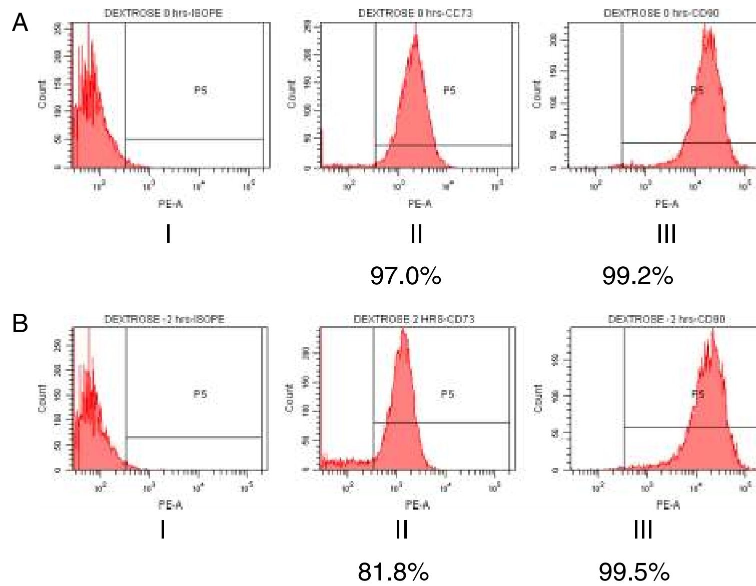

Figure 5. Immunophenotyping of MSC surface markers at 0 and 2 h suspended in 5% dextrose following the freeze–thaw cycle. (A) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 2 h. II, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 97.0% positive for CD73. III, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 2 h; the cells are 99.2% positive for CD90. (B) I, PE-conjugated isotype control for BM-MSCs suspended in 5% dextrose at 0 h. II, surface expression of CD 73 for BM-MSCs suspended in 5% dextrose at 0 h; the cells are 99.5% positive for CD73. III, surface expression of CD 90 for BM-MSCs suspended in 5% dextrose at 0 h; the cells are 81.8% positive for CD90

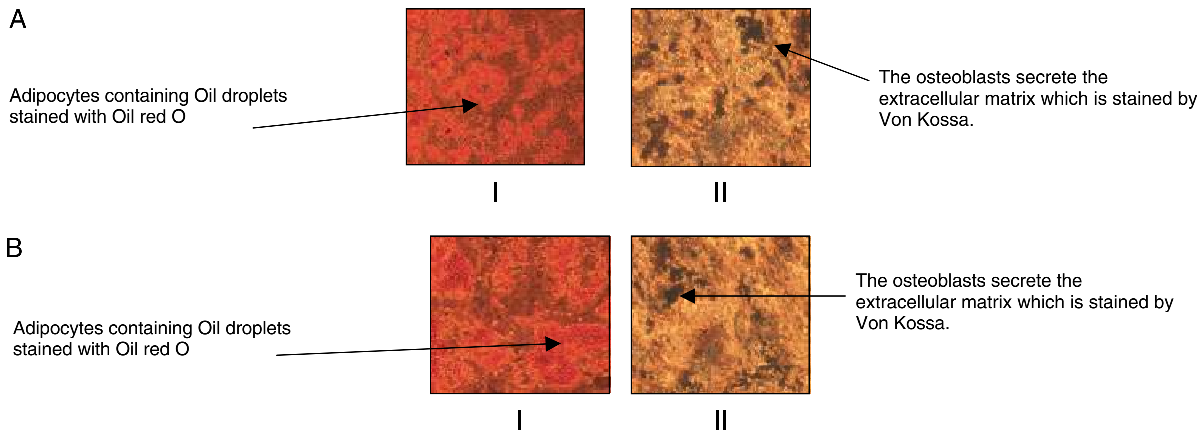

Figure 6. Differentiation capability of MSCs into (I) adipocytes and (II) osteoblasts (×10) after (A) 0 h and (B) 8 h of holding when harvested from fresh cultures. The differentiation capacity of MSCs, whether harvested at 0 h or 8 h, does not change; they equally retain the capacity to differentiate into adipocytes and osteoblasts even after 8 h of holding

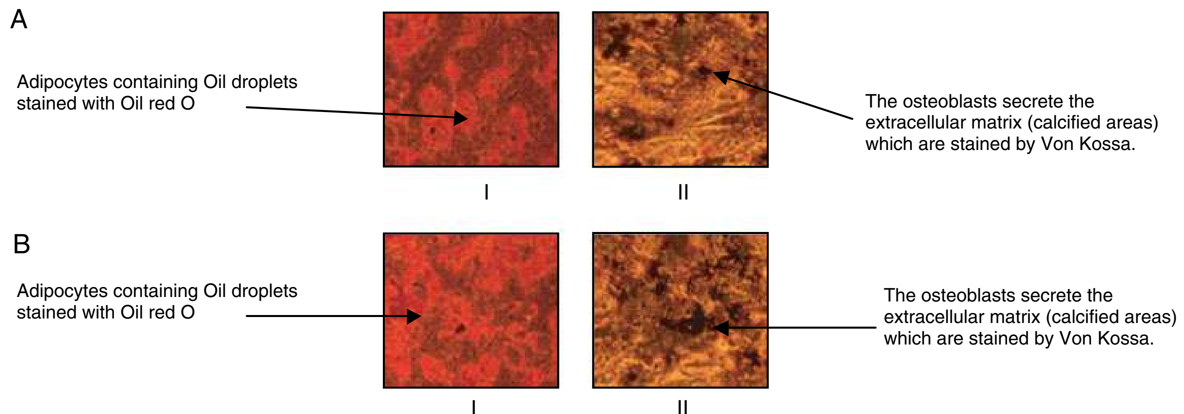

Figure 7. Differentiation capability of MSCs into (I) adipocytes and (II) osteoblasts (×10), (A) immediately following freeze–thaw (0 h) and (B) after 2 h of holding following freeze–thaw. The differentiation capacity of MSCs, whether harvested at 0 or 2 h, does not change; they equally retain the capacity to differentiate into adipocytes and osteoblasts even after 8 h of holding

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for holding the cells until they could be transplanted, as these are clinically applicable, i.e. they can be injected into patients.

In the second experiment, we used frozen–thawed MSCs and the cryoprotectant was removed by centrifugation. The results demonstrated that frozen–thawed MSCs can be held in either plasmalyte A or 5% dextrose at 4 °C for 2 h with maximum viabilities of 92.56% ± 2.63 and 91.56% ± 2.28, respectively, or at RT for 2 h with maximum viabilities of 91.73% ± 1.0 and 91.43% ± 2.51 respectively. After 4 h, the viability was found to decrease over time in all the parenteral solutions.

Transporting frozen MSCs from the central processing unit to any hospital is feasible and practical without losing their viability before thawing. Also, frozen–thawed MSCs could be used in various critical cases, such as myocardial infarction and cerebral stroke, where the period for cell transplantation is critical. However, this method has some disadvantages. While removing the cryoprotectant, the quality of the cells may be compromised and may expose the cells to an unclassified environment.

Therefore, in the third experiment, we used frozen–thawed MSCs, diluted further with plasmalyte A so as to dilute the cryoprotectant to a recommended level of administration. We demonstrated that the MSCs can be held for 2 h after thawing and dilution and can be used for transplantation without further reducing their viability. Thawing and further dilution can be done in the operation theatre, thus avoiding exposing the cells to an undefined environment and without compromising their quality. This method will also help in cases where companies are planning to develop an off-the-shelf product.

5. Conclusion

The present data show that adult bone marrow-derived MSCs can be frozen in cryocyte bags, transported to remote locations, thawed, diluted to the permissible concentration and stored for 2 h without losing their viability, immunophenotype and differentiation potential. Those cells harvested from fresh cultures can be held in 5% dextrose or saline and wait until 6–8 h before transplantation without any reduction of viability or differentiation capacity. This will help in using MSCs therapeutically in a wide variety of acute and chronic diseases, even at distant locations, without any loss of quality and also allowing companies to manufacture off-the-shelf products.

Conclusions from the experiment are as follows:

1. Cells harvested from fresh cultures can be held at 4 °C for 6 h in the solutions mentioned, while maintaining viability >90%; the viability is higher when the cells are suspended in 5% dextrose at 4 °C for 8 h.
2. Cells following freeze–thaw demonstrate a decrease in viability which was more apparent for MSCs held at 37 °C at all the time points and in all the solutions, and hence may not be a method of choice for resuspension. Also, MSCs stored in plasmalyte A (Figure 4A) and 5% dextrose (Figure 4B) after 2 h of holding and MSCs without storage showed similar patterns of expression.

The results demonstrated that plasmalyte A or 5% dextrose can be parenteral solutions of choice for frozen–thawed MSCs but should be used for transplantation within 2 h at 4 °C or RT.

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