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The sensitivity of human mesenchymal stem cells to vibration and cold storage conditions representative of cold transportation
The sensitivity of human mesenchymal stem cells to vibration and cold storage conditions representative of cold transportation

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Abstract

In the current study the mechanical and hypothermic damage induced by vibration and cold storage on human mesenchymal stem cells (hMSCs) stored at 2–8°C was quantified by measuring the total cell number and cell viability after exposure to vibration at 50Hz (peak acceleration 140m/s$^2$ and peak displacement 1.4mm), 25Hz (peak acceleration 140m/s$^2$, peak displacement 5.7mm), 10Hz (peak acceleration 20m/s$^2$, peak displacement 5.1mm) and cold storage for a number of durations. To quantify the viability of the cells, in addition to the trypan blue exclusion method, the combination of Annexin V-FITC and propidium iodide was applied to understand the mode of cell death. Cell granularity and a panel of cell surface markers for stemness, including CD29, CD44, CD105 and CD166, were also evaluated for each condition.

It was found that hMSCs were sensitive to vibration at 25Hz with moderate effects at 50Hz and no effects at 10Hz. 25Hz vibration also increased CD29 and CD44 expression. The study further showed that cold storage alone caused decrease of cell viability, especially after 48hrs, and also increased CD29 and CD44 and attenuated CD105 expressions. The cell death would most likely to be the consequence of membrane rupture due to the necrosis induced by cold storage. The sensitivity of cells to different vibrations within the mechanical system is an effect of the combination of displacement and acceleration and hMSCs with longer cold storage duration were more susceptible to vibration damage, indicating a coupling between the effects of vibration and cold storage.

Keywords— stem cells, mechanical stress, vibration, regenerative medicine, hypothermia, viability

Abbreviations:

hMSCs: human mesenchymal stem cells; hDF: human dermal fibroblasts; PI: Propidium iodide;
Introduction

Recently challenges faced during the shipping of cell and tissue therapeutic products from factory site to bedside have drawn significant attention in the emerging regenerative medicine industry (1). Traditionally transportation solutions for living microorganisms involve lyophilisation (freeze drying) or cryopreservation. However, most cell therapeutic applications require the supply of living cells for immediate and easy administration by the clinical end-user preferably without the presence of cryoprotectant (2) (3). A cold chain shipping system maintaining the temperature of products in the range of 2-8°C may be more appropriate than the frozen shipping of cryopreserved products. A survey for therapeutic product transportation (4) has identified that the two most commonly used transportation temperatures are 2~8 °C or 18~24°C (room temperature). As the scope of this work is to understand the biological consequences following current industrial practices for cold shipping and cold storage, storage and transport at 2~8°C was simulated.

Some logistics companies (5) (6) have made significant investments in packaging and container designs to maintain transport temperatures in the desired range. However in such circumstances, in addition to maintaining the temperature, the consequences of mechanical stresses on the biological system due to the vibration of the cell suspension during cold transportation must be understood. It has been demonstrated that osteoblasts (7-9) and stem cells derived from adipose tissue (10,11) and bone marrow (12) are sensitive to vibration. Depending on the frequency and amplitude of vibration and the duration of such stimulation, beneficial effects on cell differentiation have been observed, although vibration-induced cell apoptosis has also been reported (13). However, all of these studies have investigated the influence of vibrations only on cells attached on to substrates. In addition, most of the studies on the effects of vibration on cells have been conducted either under standard cell culture conditions (37°C) or at room temperature. Therefore it is not possible to directly correlate the effects of vibration reported in the literature to the situation encounters in cold transportation of cell suspensions.

While vibration effects are unlikely be an issue in the transportation of cryopreserved cells - a “block of ice” - in suspension it may be important because
a deviation from an allowed vibration dose may affect cell viability and therapeutic effect due to the sensitivity of cells to mechanical stress (14). This is analogous to the mechanical damage of cold-transported fruit as a consequence of transportation vibration, an important problem that requires careful management in agricultural transport for export (15). A survey of the literature indicates that management of transport vibrations is one of the crucial factors in the successful transportation of living organisms as goods and a number of investigations have been devoted to this subject, in particular to the impact of vibration on goods caused by various road roughnesses (16), vehicle and package resonances (17) and package handling (18). Understanding the critical levels of vibrations and forces for cellular products will assist in the design of appropriate packaging. The efficient design of appropriate packaging for cell transportation is important because over-packaging is uneconomic and raises sustainable development issues, conversely under-packaging can result in damage and loss of sales and compromised therapeutic effects leading to potential variations in the outcomes of clinical trials (19).

ASTM Standard D4169 (20) and D4728–06 (21) provide a useful review of standard practices for performance testing of shipping containers and systems and a starting point for test design. While they provide a good starting point for investigations of perishable products, living cells used as therapeutic products for implantation are significantly more complicated with higher regulatory requirements. It is therefore essential to understand the threshold of vibrations that could cause damage, the extent and recoverability of the damage, and the effect of mechanical stress on cell biological function. As human mesenchymal stem cells (hMSCs) reside in different connective tissues and in the skeletal system and contribute to tissue homeostasis, considerable research has been carried out to understand the effects of mechanical stress on the function and phenotype of hMSCs attached to substrates in vitro (22) (14) (23). It is also well understood that cells in suspension culture are subject to damage induced by hydrodynamic force due to agitation in stirred tanks (24) (25). However, the authors are aware of little work on the response of suspended hMSCs to mechanically imposed stresses other than that addressing their delivery through catheters (26). This study investigates the mechanical damage to hMSCs induced by vibration and cold storage by measuring the total cell number and viability of the cells. To establish
the threshold of vibrations that cause damage, vibrations with larger accelerations, displacements and durations than those ultimately expected in practice were applied to indicate the likelihood of damage. In order to characterise not only cell viability but also to understand the mode of cell death, in addition to the trypan blue exclusion method used in previous work (27) the cell apoptosis and death markers Annexin V-FITC and Propidium iodide (PI) were also used in this work.
Materials and Methods

As the purpose of this study was to investigate the effects of vibration induced mechanical stress on cell viability during cold transportation, the experiment was set up in a cold room with a temperature maintained at 2–8°C and vibrations emulating different transport vibration situations were used. Samples kept in the same cold storage conditions were used as a control and also for the study of cold storage effects on cell viability.

The mechanical system and vibration conditions

The mechanical system

The mechanical system (see Figure 1 (a)) used as the experimental platform was assembled by connecting a portable vibrator shaker V406 (LDS UK) to a PA 100E power amplifier (LDS UK) taking in electrical signals generated by a PC sound card. The vibration experienced by the samples was measured with an accelerometer (Bruel & Kjaer type 8301/SN 578616 –11.86pC/g) connected to a charge amplifier (Type 2635 Bruel & Kjaer). The signal from the charge amplifier was displayed on a Tektronix TDS 2012B two channel digital storage oscilloscope. The displacement, velocity and acceleration experienced by the sample were collected with time and saved to allow further analysis.

A sample holder was fabricated to hold 18 cryogenic vials containing the cell suspension. The design allowed vial sets to be retrieved or replaced with dummies during trials without affecting the balance of the holder (see Figure 1(b)). The vials were secured by a top plate (see Figure 1(c)) to prevent movement during vibration. The accelerometer was mounted in the centre of the holder and the whole assembly mounted on the shaker Figure 1(d).

Multiple frequency combination and initial single frequency experiments

Due to the lack of data within the literature on the effect of vibration on the viability of suspended cells, initial experiments using a multiple frequency combination and a range of single frequencies were carried out to identify a
working range and to establish further experimental parameters. Based on the results from previous vibration experiments using human dermal fibroblasts (HDF) (27), in the initial experiment hMSCs were exposed to vibration at multiple frequencies in order to scan for observable effects at conditions relevant to possible vehicle and package resonant vibrations. hMSCs were subjected to 24 hour repetitions of the following series of vibrations:

1) Three 20ms half sine shock pulses with a 0.98s rest interval following each shock pulse. Such a series train of pulse gave an acceleration of $140m/s^2$, displacement 5.7mm, and a repetition rate 1Hz similar to those caused by road bumps (16) and drops (18).

2) The previous vibration pattern then followed by bursts of vibration lasting three seconds with frequencies of 10, 25, 50, 100, 500, 1000, 5000Hz and with parameters defined fully in the Table 1 in order to explore the effects of a range of potential vibration parameters. In the multiple frequency experiment, a "rest" interval was needed to allow the Visual Basic program to switch between vibrations with different frequencies. This "rest" time, 50-60 ms, was the time needed to load the wav file for the next frequency.

3) Repeating 1) and 2) for 24hr.

In parallel, initial single frequency experiments were also carried out by exposing samples to vibration at 10, 25, 50, 100 and 500 Hz respectively for 24hrs (see Table 1 for vibration parameters)

*Insert Table 1 here*

**Vibration at 10Hz, 25Hz and 50Hz and cold storage experiments**

Following on the initial single frequency experiment, 25 and 50Hz were chosen respectively as representative of conditions with strong and moderate effects as observed in the initial experiment. In order to indentify the effects of duration of vibration on cell viability, exposure of hMSCs to vibration of the chosen frequencies were scheduled at 24hrs or 48hrs. To further understand the effects of pre-vibration cold storage, some cell suspensions were kept in cold storage for up to 144 hrs before being vibrated at 50Hz. Investigation of cold storage effects without vibration were also carried out in a parallel study by measuring cell viability for samples collected at 24hr intervals during 96hr cold storage. The
details of vibration parameters, including displacement, acceleration, velocity and energy input were listed in Table 1. A high value of acceleration, 140m/s$^2$, was chosen for vibration at 25 and 50Hz in order to include conditions representative of not only challenging transport situations but also to allow the simulation of potential nonlinear parametric sloshing phenomena, such as rotational motion, chaotic motion and free surface disintegration (28). To further identify the vibration parameters leading to the cell damage observed at 25Hz, cells were also exposed to a 10Hz vibration with a similar displacement to that at 25Hz but with a much reduced acceleration (see Table 1). The acceleration for the 10Hz vibration was necessarily reduced to 20m/s$^2$ from 140m/s$^2$ as sine wave frequency $f$, displacement $x$ and acceleration $a$ are connected via the expression

$$a = (2\pi f)^2 x$$

and it is consequently impossible to have similar accelerations and displacements at 10Hz to those at 25Hz for this mechanical system.

**Cell suspension preparation**

Human mesenchymal stem cells (hMSCs) harvested from normal human bone marrow and cultured to passage 2 (Lonza) were used as cell sources to establish a working cell bank at passage 4 sufficient to allow the use of similar input cells for all experiments. Cells were seeded at a density of 1900 cells/cm$^2$ and maintained in T-175 culture flasks. The culture medium used was Lonza human mesenchymal stem cell growth medium (MSCGM) supplemented with mesenchymal cell growth supplement (MCGS, Lonza). During culture the medium was changed every 2-3 days to ensure the presence of sufficient nutrients and when the culture reached 80-90% confluence cells were removed from the surface of the culture flasks by trypsinisation. Samples for the vibration experiments were prepared by aliquoting 1ml of cell suspension in cell culture medium with a concentration of $10^6$cells/ml into a 2ml sterile cryogenic vial (Nalgene) with a 10mm headspace. To reflect those conditions anticipated for cell transport as a therapeutic, no hypothermic storage media was used during cold storage so that no manipulation of the supplied cells would be needed to remove the hypothermic media at clinical sites such that the received products can be delivered to the patient. The vials
loaded with cell suspension were capped, sealed and mounted on the shaker and corresponding control samples were also kept in the same environment.

**Cells viability evaluation and surface antigen expression analysis**

Cell viability and surface antigen expression were analysed immediately after the vibration and cold storage experiments. Two automated cell counting systems based on the trypan blue exclusion method were used. The first, the Cedex® (Innovartis) was suitable for counting cells at high concentrations but required the whole sample volume (29); the other, the Countess® Automated Cell Counter (Invitrogen) only required 20µl sample volumes and consequently allowed further analysis of mode of cell death for the same sample.

The cell suspension was mixed by repeated gentle pipetting to break up any clumps and to redistribute the cells throughout the medium evenly. For the trypan blue staining cell suspension was mixed with an equal volume of 0.4% trypan blue and cells were counted immediately using the built in image analysis software of the Cedex or Cell counter in order to achieve accurate cell number measurements minimising the effects of cell clumping.

Cell viability measured by trypan blue exclusion method revealed the percentage of cells without an intact membrane alone. In order to further understand whether the loss of membrane integrity was attributable to the vibration-induced mechanical stress, or other mode of cell death, samples vibrated at 10, 25 and 50Hz and exposed to different cold storage duration were further analysed by both the trypan blue exclusion method and flow cytometer apoptosis analysis (Beckman Coulter Cell Lab QuantaSC Flow Cytometer). For Annexin V apoptosis analysis, following the protocol from the supplier, a combination of Annexin V-FITC and Propidium Iodide (PI) (Invitrogen, UK) allows the distinction between early apoptotic cells (Annexin V+), apoptotic (Annexin V+ and PI+), dead cells (PI+) and viable cells (unstained).

As it has been reported that re-warming of cells taken from cold storage resulted in apoptosis due to the activation of the apoptotic process for cells partially injured by cold stress (30), samples were immediately analysed after cold storage and vibration experiments to avoid introduction of any further cellular stress due to re-warming. This reflects the timing and conditions for handling of such a cellular therapeutic product in clinical practice.
Samples were centrifuged at 300g for 5 minutes and directly conjugated antibodies were added after re-suspension at the manufacturer’s recommended concentrations, followed by incubation at room temperature for 45 minutes in the dark. Cells were also incubated with monoclonal antibodies specific to mesenchymal stem cell surface markers CD29 (FITC), CD44 (FITC), CD105 (PE), CD166 (PE) (Beckman Coulter UK). All monoclonal antibodies were used in conjunction with their respective isotype controls and autofluorescence and non-specific binding of the cells obtained with isotype control were used as negative controls. For each sample run 10,000 events within the population of interest were recorded and analysed cell populations of interest were gated according to side scatter (SS) and particle size. Analysis of a cell suspension prepared on day 0 was used to set the voltage to the appropriate level to obtain a signal for both autofluorescence and non-specific binding below the first log decade of the fluorescent signal axis and the setting established used for the rest of the experiments. Cyflogic version 1.2.1 (CyFlo Ltd., Turku, Finland) was used to display the profiles of the negative control and samples as an overlay histogram.

**Analysis of significance of experimental data**

Samples for all experiments were prepared in triplicate and data were normalized and presented as the ratio between the vibrated and the control samples. In order to establish whether there were statistically significant differences between the results obtained from different conditions, the confidence interval of the ratio of two means (31) were calculated and presented as error bars on plotted data. This gives 95% percent confidence that vibrated samples are significantly different from the controls if the line crossing vibrated/control = 1, representing the null hypothesis that the vibrated and the control samples are exactly the same, is outside the error bar.
Experimental Results

Multiple frequency combination and initial single frequency experiments

24 hours of vibration with the multiple frequency combination and vibration at a single frequency of 25Hz caused the largest decrease in total cell population and cell viability and (see Figure 2 a and b). Moderate effects were observed in the 50Hz single frequency experiment and as vibration frequencies increased from 100 to 500 Hz and also at 10Hz the decrease in cell viability and total cell numbers due to vibration were less apparent. To further confirm and verify the effects noticed, experiments at 10, 25 and 50Hz were repeated with controlled cold storage duration and vibration duration.

Vibration at 50Hz, peak acceleration 140m/s², peak displacement 1.4mm

Insert Figure 3 here

Total cell numbers and cell viability relative to the control showed a small, but not statistically significant decrease (See Figure 3 a) for both 24 and 48hrs vibration with no pre-vibration cold storage. However when samples were stored for 144hrs before being exposed to 24hrs of vibration, the ratios of the total cell numbers and cell viability between the vibrated to the control samples were dramatically reduced in comparison to those without pre-vibration cold storage (Figure 3a) and significantly lower than 1 for total cell number and viability.

Further flow cytometer analysis of sub-populations at different stages of cell death revealed no significant difference in viable cell population between the vibrated and the control (see Figure 3 b), and between 24 and 48hrs vibration. Trends indicating a presence of more apoptotic and dead cells were observed after 24hrs and 48hrs vibration. The percentage of dead cells after 48hrs of vibration was significantly greater when compared to the control (see Figure 3 b). As the percentages of early apoptotic and apoptotic cells were much smaller than those of viable cells for both 24 and 48hrs (see Figure 3 c), it is not unexpected that the error bar based on the normalized data appears large even for small variations.
between the samples within the groups. When both viable and early apoptotic (PI negative), and dead and apoptotic (PI positive) components of the populations were combined (see Figure 3 d), the viability measured relative to the control is similar to that quantified by the trypan blue exclusion method (see Figure 3 a).

**Vibration at 25Hz, peak acceleration 140m/s^2, peak displacement 5.7mm**

*Insert figure 4 and 5 here*

Trypan blue measurements (Figure 4a) showed that with vibration total cell numbers and cell viability reduces to 4% and 28% of the control respectively. Flow cytomeric dot plots of vibrated samples and the control (Figure 5) revealed that most of the cells analysed were propidium iodide positive but Annexin V negative after exposure to vibration, indicating the loss of membrane integrity and subsequent cell death. Cells without exposure to vibration were scattered across the four regions of the dot plot with more of the population in the apoptotic region (both Annexin V and propidium iodide positive). Further quantitative analysis of the flow cytometry measurements (Figure 4b) indicated that the proportion of the dead cells in the vibrated samples was more than ten times that in the control. It also showed moderate decrease in the percent of viable and early apoptotic cells, but the percent of apoptotic cells was much less than that observed in the control. When both viable and early apoptotic cells, and apoptotic and dead cells, were combined (see Figure 4 c), the percent of dead cells was still 3-4 times of that in the control and the percent of viable cells was ~ 68% of the control.

**Vibrations at 10Hz, peak acceleration 20m/s^2, peak displacement 5.1mm**

*Insert Figure 6 here*

Vibration at 10Hz tended to decrease total cell number and cell viability slightly, but the observed change was not significant relative to the control (Figure 6a). When the subpopulations of viable and early apoptotic, and dead and late apoptotic cells (Figure 6 b), were combined, there was still no significant
difference between the vibrated sample and the control as shown by the outcomes of measurement using the flow cytometer.

**Effect of cold storage**

The results of flow cytometry measurements to quantify the effect of up to 96 hrs of cold storage on cell viability are presented in Figure 7. This shows that the number of dead cells (PI+) in the population increased slightly after 24 hrs, however from 48 to 72 hrs a dramatic increase of dead cells from 20% to nearly 40% of the cell population was observed. The percentage of early apoptotic and apoptotic cells did not change significantly. Further viability measurements using the Countess® by the trypan blue method (Figure 7(b)) agreed with the flow cytometry measurements (Figure 7(a)), particularly for 72 and 96 hours of the cold storage.

*Insert Figure 7 here*

**Evaluation of cell surface markers**

*Insert Table 2 here*

The hMSCs at T0 were positive for CD29, CD44, CD105 and CD166, with the strong expression of CD105 and CD44 and moderate expression of CD29 and CD166. After 24 hrs cold storage, moderate expression of CD29 and CD44 was still noticed, but no expression of CD105 and CD166 could be detected. As the cold storage duration increased to 48 and 72 hrs, the expression of CD29 and CD44 became more significant and was ultimately, at 96 hrs, above the range set for the instrument. Moderate expression of CD105 and CD166 was shown after 48 and 72 hrs cold storage, but at 96 hrs only moderate CD166 expression was observed and CD105 expression was negative. When cells were vibrated at 10 Hz, there was little difference in CD marker expression to cells solely kept in cold storage. For vibrations at 25 Hz, the expression of CD29 and CD44 was higher than the control. The side scatter also indicated a decrease of cell granularity with prolonged cold storage with and without vibration. After 24 and 48 hrs of cold storage, the side scatter showed a bimodal distribution indicating a mixture of populations of normal cells and cells with reduced granularity. The latter
population increased with storage duration as evidenced by the increase of the intensity of the left hand peak. When cold storage was prolonged over 72hrs, the bimodal distribution was no longer observed and the single distribution illustrated the dramatic decrease of cell granularity.
Discussion

With the development of cell/tissue-based regenerative medicine products for clinical application, it has become more critical to establish a supply route to ensure the quality of these products and, hence, the success of their clinical application. Different transportation strategies have been investigated (32) and a significant amount of research has been carried out to understand the effects of cold storage and cryopreservation on the viability of cells (33) (34) (35). The study carried out within the research reported here has investigated the effects of the combination of cold storage and mechanical stress during cold transport of a hMSC suspension without the presence of cryoprotectant.

Consequences of vibration on cell viability

Within the initial preliminary experiment, the dramatic reduction on cell viability were noticed for hMSCs, consistent with that observed also for human dermal fibroblasts (27). The single frequency sweep experiment further revealed that the effects on cell viability were related to the parameters of the vibration, including frequency, displacement and acceleration rate. Findings from this initial experiment led to further investigations of changes of cell viability, total cell number and status of cell death induced by cold and mechanical stress when hMSCs with different pre-vibration cold storage durations were exposed to vibration conditions of specified parameters.

When the hMSC suspension was vibrated at 50Hz, no significant effects on cell viability were noticed for cells vibrated immediately after preparation of the suspension. However, for cells stored for 144 hrs in cell culture medium at 2~8°C a significant reduction of cell viability was observed. This showed that cells exposed to prolonged cold storage were more susceptible to the mechanical stress induced by vibration.

At 25Hz, dramatic reductions in cell number and cell viability were observed. The Trypan blue exclusion method showed a dramatic reduction in viability to 28% of that of the control, while the Annexin and PI method gave a result of 68% of that of the control. As the total cell number was much reduced after vibration, the difference was likely due to the lack of reliability of the trypan blue exclusion method at lower cell densities (36).
Comparison of the results of 24 hours vibration at 25Hz and 50Hz showed that the conditions experienced at 25Hz reduced the final total cell number to 0.4% of the control, but that the total cell number was still around 95% of the control for 50Hz. A similar response to vibration at different frequencies were also observed in previous experiments with hDF (27). The differences between the vibration parameters of these two conditions include frequency and displacement. In order to further understand the influence of displacement on cell viability, an experiment was also carried out at 10Hz with a displacement similar to that for 25Hz and also at 100Hz with a much reduced displacement and similar acceleration. There was no significant effect on cell viability when samples were vibrated at 10 and 100Hz, indicating the dramatic and moderate reduction on cell viability observed at 25Hz and 50Hz respectively may be the consequences of combinational effects of displacement and acceleration.

**Mode of cell death induced by vibration and cold storage**

The influence of cold storage alone on cell viability and the function of cell therapeutic products has been extensively investigated, especially for hematopoietic stem cells derived from different tissues (37) (38) (33) and regenerative medicine products based on cord-/bone marrow-derived mesenchymal stem cells (39) (40)(41) Solely considering the effects of cold storage, the evaluation of cell membrane integrity and apoptosis showed an increase of both Annexin V+ and PI+ cell populations, especially after 24 and 48hrs cold storage, indicating cells are approaching cell death. The increase of the PI+ cell populations was further observed with a cold storage duration extended from 2 to 4 days, consistent with those reported for hypothermic conservation of hematopoietic progenitors (42). Annexin V has been generally used as an indicator to identify apoptotic cells. However, Annexin V expression is not exclusive to apoptotic cells as translocation of phosphatidylserine to the external cell surface also occurs during cell necrosis and oncosis (43) (44). Researches on cold storage effects have shown that cold storage alone only resulted in mainly necrosis (30) (45). Hence the observed increase of Annexin V+ and PI+ cell populations with prolonged cold storage duration could indicate more cells
proceeding into cell death through oncotic necrosis (also referred to as swelling necrosis), a form of passive or accidental cell death. Although cell apoptosis induced by hypothermia/re-warming has been investigated extensively, especially for tissue preservation of major organs, such as liver or kidney tissues, there are still different opinions on whether cell death induced by hypothermia is through the mode of apoptosis (46) or necrosis (47). Hence, the involvement of apoptosis in cold-induced cell death could not be excluded totally (45), further characterization, perhaps using an apoptosis DNA fragment test, is needed to identify and confirm the mode of cell death. In addition, the final stage of apoptosis, e.g. caspase activation, can only occur if the cells are metabolically active, i.e. returned to culture. Future research should therefore apply a real-time biochemical indicator of cell injury, e.g. heat shock proteins (48,49), to distinguish between the factors of vibration, cold storage and re-warming and their contribution to necrosis or apoptosis.

The effects of the addition of vibration to the cell samples shown at 50Hz 48hrs vibration, a transition from expression of Annexin V+ to an expression dominated by PI+ was observed. The transition of cell population would further imply that as a consequence of cold-induced cell oncosis and eventually necrosis, prolonged cold storage induces change of cell membrane and causes the cells to be more susceptible to vibration damage. The synergistic effect of cold storage and vibration was further confirmed by the dramatic reduction on cell viability and total cell number observed for samples with 144hr pre-vibration cold storage. These cold storage effects on the cell membrane were also verified further by the rapid decrease of cell side scatter, indicating the decrease of cell granularity due to the damage to the cell membrane and the subsequent release of cell organelles. Though cell granularity also diminishes as apoptosis proceeds, considering there was no further incubation to activate apoptosis before sample testing, the reduction of cell granularity would most likely to be the consequence of membrane rupture due to the necrosis induced by cold storage (50).

**Influence of cold storage and vibration on stem cell surface markers**

The panel of mesenchymal stem cell surface markers investigated also revealed some sensitivity of the cells to the influence of cold storage and vibration...
conditions. Prolonged cold storage durations intensified CD29 and CD44 expression, attenuated CD105 expression and had no obvious effects on CD166. CD105, also called endoglin, is involved in angiogenesis and has been suggested as an appropriate marker for tumor-related angiogenesis (51). Recently Levi et. al. have identified the correlation of low expression of CD105 with an increased osteogenic potential for adipose-derived stromal cells (52). Considering that stem cells are more resistant to hypothermic environments than other somatic cells (53), the change of cell surface markers observed could be attributed to a subpopulation of hMSC surviving in the cold storage conditions. This finding is in accord with those observed by others that the tolerance of cells to cold storage is dependent on cell type (54) (55) and that only a certain sub-population of cells will survive prolonged cold storage. (33).

Increased CD29 and CD44 expression was noticed after 24hr of 25Hz vibration, but not at 10Hz. Heng et.al have reported some non-significant change of gene expression of hMSCs phenotypic markers, depending on the shear stress experienced by cells during trans-catheter injection (26). Considering the relatively large displacement and acceleration at 25Hz, the shear stresses that hMSCs have been exposed to could be much higher in this study. Moreover, the flow of medium within the vial during vibration may be more complex than the laminar flow in a catheter and also the duration of exposure to the hydrodynamic-induced shear stress was longer in the current study. It has been reported that CD44 positive haematopoietic progenitor cells can sense shear stress in laminar flow (56) and that the response of osteoblast-like cells to oscillatory shear stress was through the activation of CD29 (beta1 integrin) (57). The increase of CD29 and CD44 expression at 25Hz noticed in this study could be due to the higher shear stresses experienced by the cells as the consequence of different hydrodynamic behavior within the system at the specified frequencies. Similar differing effects for shear stresses generated by laminar flow and orbital liquid motion have been reported for endothelial cells (58). It is anticipated that the higher CD29 and CD44 expression may facilitate the binding of hMSCs with extracellular matrix and, hence encourage cell migration, recruitment and tissue regeneration after locally/systematic administration. (59) (60)
Correlation of vibration parameters with real-life cold chain shipping practice

Initially two "extreme" examples derived from the publications of Litak and Garcia were used as a benchmark to aid the establishment of vibration parameters. Litak et al reported shock pulses of 100ms durations with peak accelerations of \(20\,\text{m/s}^2\) or \(50\,\text{m/s}^2\) with or without damping when a van was crossing a rail track at 20km/h (16). Garcia et al. also reported shock pulses with a maximum peak acceleration of \(850\,\text{m/s}^2\) and a duration of 20ms when mid-sized (0.36m × 0.34m × 0.34m) and lightweight (6.5kg) packages were shipped between Europe and the USA (18). As the maximum displacement allowance of the shaker in the experimental arrangement used was 7mm, it was not possible to simulate the acceleration rate and duration of these two extreme examples (note that 2cm or 5cm displacements are needed for \(20\,\text{m/s}^2\) or \(50\,\text{m/s}^2\) accelerations and 3.5cm for \(850\,\text{m/s}^2\)). Also considering the cost of cell therapeutic products and the high patient risk if malfunctioning products were to be used, the worst case must be investigated in order to satisfy the demanding regulatory requirement. The 12kg mass packaging system used (Greenbox)(5) was designed to maintain cold chain temperatures during transportation and had a lower anti-shock performance when compared with the 6.5kg mass system used in Garcia’s study specially designed to control the effects of vibration. Hence, the vibration parameters chosen were a compromise between the vibration conditions of the two extreme examples and experiments were carried with the maximum accelerations achievable by the experimental system used.

At the maximum displacement of 6mm, a 20ms shock pulse with an acceleration of \(140\,\text{m/s}^2\) could be generated. To identify the threshold of vibrations that could cause cell damage, vibration tests at different frequencies were carried out at acceleration rate \(140\,\text{m/s}^2\) whenever possible. For 10Hz vibrations the maximum acceleration is \(20\,\text{m/s}^2\) (similar to that reported by Litak) and displacement is 5mm, and these are similar to those used in the random vibrations test for shipping containers defined by ISO 13355 standard (21). An acceleration of \(140\,\text{m/s}^2\) and duration 20ms also represent the effects of some drops and disturbances caused by road bumps and trenches, especially when the vehicle speed is greater than 20km/h. The destructive effects of vibration noticed in this study imply that cell damage is more likely to occur in such cell suspension
containing packages when vibrated at higher amplitudes during cold transportation.

In summary, with the development of stem cells and cell culture technology, increasing attention has been placed on packaging design and on identifying product dispensing routes facilitating the clinical end use. The findings reported here on the effects of vibration and cold storage on cell viability and total cell number highlight the importance of understanding the sensitivity of cells to their mechanical and thermal environment during transport and the necessity of improved package design for highly regulated cell/tissue-based therapeutic products. Currently the evaluation and validation process for any specified format of shipping container purely depends upon in vitro and in vivo characterization of the shipped cells. Further work will concentrate on understanding the hydrodynamics of medium flow in the system to identify the thresholds for effects and to understand the underlying mechanisms giving rise to the observed biological consequences. This will inform a more rational and active approach to package design for cellular products, allowing decision making based on real time objective data rather than purely relying on consequence evaluation using cell characterization techniques to ensure the consistency and stability of the products following transport from the factory site to the bedside.

**CONCLUSIONS**

Flow Cytometry and trypan blue exclusion measurements indicated that cold storage alone in cell culture medium caused decreases of cell viability, viable and total cell numbers, especially after 48hrs. Cell death is most likely to be the consequence of membrane rupture due to the necrosis induced by cold storage. The sensitivity of cells to different vibration conditions within the mechanical system is a combination of the effects of displacement and acceleration and hMSCs with longer cold storage duration are more susceptible to vibration damage, indicating a coupling between the effects of vibration and cold storage.

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References:


Table 1

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<th>Frequency [Hz]</th>
<th>Max. Displacement [m]</th>
<th>Max. acceleration [m/s²]</th>
<th>Max. velocity [m/s]</th>
<th>Energy ~ [max.velocity]^2 [m²/s²]</th>
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Figure 1

(a) Diagram of experimental setup:
- DESK TOP PC
- Sound card
- PA 100E Power oscillator
- Sample holder with samples
- Digital storage oscilloscope
- Charge amplifier
- V406 LDS shaker
- Accelerometer
- Final data saved at USB memory stick

(b) Photograph of sample holder

(c) Photograph of digital storage oscilloscope

(d) Photograph of charge amplifier
Figure 2.

(a) Total cell numbers vibrated/control vs. frequency

(b) Cell viability vibrated/control vs. frequency
Figure 3.
Figure 5

[Diagram showing a scatter plot with quadrants labeled Dead, Apoptotic, Viable, and Early apoptotic. Two plots are shown side by side.]
Figure 6

(a) Total cell number and viability after storage for 24 hours and vibration for 24 hours.

(b) Viable and early apoptotic cells, and apoptotic and dead cells after storage for 24 hours and vibration for 24 hours.
Figure 7

(a) Graph showing cell percentage over the duration of cold storage in hours. The graph indicates the percentage of dead, apoptotic early, and apoptotic cells.

(b) Graph showing the percentage of viable and dead cells over the duration of cold storage in hours. The graph highlights the decrease in viable cells and increase in dead cells over time.