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**Alkylating chemotherapeutic agents cyclophosphamide and melphalan cause functional injury to human bone marrow-derived mesenchymal stem cells**

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## **Abstract**

The adverse effects of melphalan and cyclophosphamide on hematopoietic stem cells are well known, however the effects on the mesenchymal stem cells (MSCs) residing in the bone marrow are less well characterized. Examining the effects of chemotherapeutic agents on patient MSCs *in vivo* is difficult due to variability in patients and differences in the drug combinations used, both of which could have implications on MSC function. As drugs are not commonly used as single agents during high dose chemotherapy (HDC) regimens there is a lack of data comparing the short or long term effects these drugs have on patients post treatment. To help address these problems the effects of the alkylating chemotherapeutic agents cyclophosphamide and melphalan on human bone marrow MSCs were evaluated *in-vitro*. Within this study the exposure of MSCs to the chemotherapeutic agents cyclophosphamide or melphalan had strong negative effects on MSC expansion and CD44 expression. In addition, changes were seen in the ability of MSCs to support hematopoietic cell migration and repopulation. These observations therefore highlight potential disadvantages in the use of autologous MSCs in chemotherapeutically pre-treated patients for future therapeutic strategies. Furthermore, this study suggests that if the damage caused by chemotherapeutic agents to marrow MSCs is substantial, it would be logical to use cultured allogeneic MSCs therapeutically to assist or repair the marrow microenvironment after HDC.

**Key Words:-** Mesenchymal Stem Cells, Transplantation, Chemotherapy, Hematopoietic Stem Cells, Bone marrow

## Introduction

The biological functions of bone-marrow derived mesenchymal stem cells (MSCs) *in vivo* include both hematopoietic support and tissue maintenance. These functions are achieved through MSCs having a multipotent capability to generate progeny that can differentiate down multiple cell lineages to form bone, cartilage, fat cells, and bone-marrow stroma [1], in addition to having the apparent ability to support the expansion of primitive hematopoietic cells through the expression of a variety of cytokines and the reconstruction of the hematopoietic microenvironment [2-4].

Hematopoietic recovery after high dose chemotherapy (HDC) in the treatment of hematological diseases may be slow and/or incomplete. This is generally attributed to progressive hematopoietic stem cell failure, although defective hematopoiesis may be in part due to poor stromal function. HDC and irradiation used with or without autologous hematopoietic stem cell (HSC) rescue in the treatment of hematological malignancies and other cancers may cause long lasting damage to bone marrow stromal cells, thus impairing hematopoiesis and may have a possible involvement in slow or poor engraftment post HSC transplantation.

Patients who have undergone HDC commonly display disruption of the marrow architecture with hemorrhaging, loss of fat, and loss of stromal compartments [5]. Studies have also demonstrated that a recipient's stromal cells are damaged after allogeneic stem cell transplantation [6-8], or even from chemotherapeutic drugs alone [6-15]. We have also recently demonstrated a significant reduction in MSC expansion and MSC CD44 expression by MSCs derived from patients receiving HDC regimens for hematological malignancies, thus implicating potential disadvantages in the use of autologous MSCs in chemotherapeutically pre-treated patients for future therapeutic strategies [16]. These effects are relevant not only in patients treated with HDC without allogeneic stem cells but also in recipients of allogeneic stem cell transplants in whom bone marrow stroma remains recipient in origin after the transplant [17-19]. If damage caused by chemotherapeutic agents to marrow MSCs is substantial, it would be logical to use cultured allogeneic MSCs therapeutically to assist or repair the marrow microenvironment after HDC. Evidence from animal experiments [20-23] and clinical trials [24] suggests that co-transplantation of cultured allogeneic MSCs may have a role in facilitating hematopoietic stem cell engraftment after stem cell transplants, although the biological mechanisms involved are unclear.

Examining the effects of chemotherapeutic agents on patient MSC *in vivo* is difficult due to patient variability and differences in the drug combinations used, both of which could have implications on MSC function. With drugs not being commonly used as single agents during HDC regimens there is also a lack of data comparing the short or long term effects that these drugs have on patients post treatment. This *in-vitro* study was therefore designed to help address these problems using the alkylating agents cyclophosphamide and melphalan at clinically relevant concentrations to evaluate the effects of chemotherapeutic exposure *in-vitro* on the functional properties of femoral-shaft marrow MSC cultures.

## **Methods**

### ***Femoral shaft bone marrow collection***

Bone marrow samples were obtained by an Orthopaedic surgeon at the Avon Orthopaedic Centre (AOC), Southmead Hospital, Bristol, with informed written consent and hospital ethics committee approval. Bone marrow was removed from the femoral shaft during surgery for total hip replacement to make space for the prosthetic joint and placed into a sterile 50ml tubes containing 1000 I.U heparin. Patients with a history of malignancy, immune disorders or rheumatoid arthritis were excluded from the study. Femoral shaft bone marrow donors were healthy apart from osteoarthritis, and were not receiving drugs known to be associated with myelosuppression or bone marrow failure.

### ***Umbilical cord blood sample collection***

Umbilical cord bloods were obtained by midwives in the Central Delivery Suite, Southmead Hospital, Bristol, with maternal written informed consent and local hospital ethics committee approval. Cord blood samples were collected by gravity into sterile 50ml tubes containing 1000 I.U heparin after the umbilical cord had been clamped and cut. All samples were from normal full-term deliveries, and collection was entirely at the discretion of the midwife in charge.

### ***Establishment of mesenchymal culture***

Femoral shaft marrow samples were broken up with a scalpel and washed with Dulbecco's Modified Eagles Medium (DMEM) until remaining material (bone) looked white at the bottom of the 50ml tube. All washings were pipetted into a new 50ml tube and kept for centrifugation. The suspension was centrifuged and re-suspended in DMEM. Marrow aspirates were overlaid onto an equal volume of Lymphoprep™ (Axis-Shield, Dundee, UK) (density 1.077+/-0.001g/ml) and centrifuged at 600g for 35mins at room temperature to separate the mononuclear cells (MNC) from neutrophils and red cells. The mononuclear cell (MNC) layer was harvested and washed twice in DMEM Sigma-Aldrich, UK).

### ***MSC culture***

Isolated MNCs were centrifuged and resuspended in MSC medium (DMEM with 10% FCS selected for the growth of MSCs (StemCell Technologies, London, UK), and 1% Penicillin and Streptomycin (Sigma-Aldrich, Gillingham, UK)). Vented flasks (25cm<sup>2</sup>) containing 10ml of MSC medium were seeded with 1x10<sup>7</sup> nucleated cells (seeding density = 400,000 cells/cm<sup>2</sup>) for passage 0. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and non-adherent hematopoietic cells removed by media exchange after 3-5 days. Cells were then cultured for two weeks at passage 0 and fed by half medium exchange.

To calculate MSC expansion, the adherent cells were resuspended using 0.25% trypsin (Sigma-Aldrich, Gillingham, UK) and re-seeded at 7.5x10<sup>4</sup> cells per flask (seeding density = 3000 cells/cm<sup>2</sup>) into passage 1. Thereafter, the cells were re-plated at 7.5x10<sup>4</sup> cells per flask (seeding density = 3000 cells/cm<sup>2</sup>) every 14 days for up to 5 passages. During this time cells were fed every week with MSC medium by half medium exchange.

### ***Immunophenotyping MSC cultures***

To ensure a homogenous population of MSCs had been cultured immunophenotyping of surface markers, using flow cytometric techniques, was carried out according to previous reports [1, 25-26]. MSCs were examined using the fluorescently tagged monoclonal antibodies anti-CD105, -CD45 -CD166, -CD44, -CD29 (BD Biosciences, Oxford, UK). For immunophenotypic analysis, MSCs were detached from culture flasks at second passage using 0.25% trypsin for 5 min, washed with PBS to remove trypsin, and resuspended in MSC medium at 10<sup>6</sup> cells/ml. The cell suspension was incubated in the dark at 4°C for 30 minutes with the specific monoclonal antibody. Cells were then washed with DMEM, centrifuged at 400g for 5mins, and resuspended in MSC medium for analysis. At least 10,000 events were analyzed on a BD FACS vantage SE and analyzed with CellQuest™ software (BD Biosciences, Oxford, UK). Gates were set on the analysis to remove cellular debris.

### ***Differentiation***

Mesenchymal stem cells were induced into adipogenic, osteoblastic and chondrogenic differentiation by culturing identical numbers of MSCs, at second passage, in NH Adipodiff medium, NH Osteodiff medium and NH Chondrodifff medium (Miltenyi Biotec, Surrey, UK) respectively according to the manufacturer's instructions. Differentiation of MSCs was only performed prior to any *in vitro* treatment with drugs, as differentiation was used solely for the purpose of MSC characterisation. Adipogenic differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O. Osteogenic

differentiation was visualized morphologically and also by the presence of calcium stained with Alizarin red. Finally chondrogenic differentiation was characterised by the production of the extracellular matrix proteoglycan aggrecan, visualized using immunofluorescent detection by labeling of aggrecan using a mouse anti-human aggrecan (4F4) antibody (Santa Cruz Biotechnology, Heidelberg, Germany).

### ***Chemotherapeutic agents***

The following chemotherapeutic agents were tested: Melphalan (50  $\mu\text{mol/L}$ ) or Cyclophosphamide (500  $\mu\text{mol/L}$ ) with the addition of S9 extract (0.4mg/ml) (Sigma-Aldrich, UK). Cells prior to each experiment were incubated with each treatment for 48 hours in DMEM, 10% FCS. The concentrations used for each chemotherapeutic agent used were determined from published data of plasma concentrations from patients undergoing intensive high-dose chemotherapy treatment or pre-stem cell transplant conditioning [27-31]. S9 extract contains the cytochrome P450 proteins that are an essential group of enzymes involved in the metabolism of drugs and chemotherapeutic agents [32]. Most cell cultures contain little, if any, cytochrome P450 mixed function oxidase metabolic capability [28], therefore S9 extract was used as a supplement in culture as cyclophosphamide requires systematic bioactivation by cytochrome P450 into its active cytotoxic compound 4-hydroxycyclophosphamide which forms both phosphoramidate mustard and acrolein [33]. The effects of S9 extract alone on experimental conditions was investigated in all cases and not shown to be significantly different from control conditions (data not shown).

### ***Cytotoxicity assay***

Cell viability after chemotherapeutic insult was detected using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, UK), according to the manufacturer's instructions. Prior to treatment cells were plated at 750 MSCs/well in 100 $\mu\text{l}$  of MSC media and were dispensed into 96-well plates in triplicate and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> until cultures had reached confluence.

### ***Immunoblotting for MSC CD44***

At 48 hours post treatment cells were lysed using Beadlyte cell signalling universal lysis buffer (Upstate™, UK). The 2-D Quant Kit™ (GE Healthcare) was then used to quantify the concentration of total protein within each cell lysate sample according to manufacturer's instructions to ensure equal loading of cell lysates. Lysates



were heated to 95°C for five minutes with Laemmli 2x sample buffer (Invitrogen, UK) and run on NuPAGE Novex 4-12% Bis-Tris Zoom gels (Invitrogen). After transfer to PVDF membrane (Bio-Rad, UK) and blocking in 5% w/v powdered milk, membranes were incubated overnight in primary antibody at 4°C (in Tris-buffered saline/5% bovine serum albumin). The antibody used was CD44 antibody (BRIC235 obtained from the IBGRL, Bristol) diluted 1:1000 (v/v) in PBS-Tween, 5% BSA. Immunoreactivity was detected using secondary anti-rabbit horseradish peroxidase conjugated antibodies (Abcam, UK) (in Tris-buffered saline/5% bovine serum albumin) and specific protein expression patterns were visualized by chemiluminescence using an Amersham ECL Plus™ Western Blotting Detection System (Amersham, UK).

#### ***Purification and flow cytometric analysis of CD34<sup>+</sup> cord blood cells***

CD34<sup>+</sup> cell isolation was undertaken after MNC separation as previously described in the establishment of MSC culture. CD34<sup>+</sup> cells were isolated from the MNC fraction obtained from cord blood harvests using the immunomagnetic MiniMACS (Magnetic-Activated Cell Sorter) CD34 isolation system according to the manufacturer's instructions (Miltenyi Biotec, UK). The CD34<sup>+</sup> cells obtained from the MACS positive fraction were then assessed by cell counting and flow cytometry as described in the 'assessment of peripheral blood contamination of marrow MNC harvests' section above, with the exception that cells were assessed for CD34<sup>+</sup> content by labelling with anti-CD34 clone HPCA-2 (BD Biosciences, Oxford, UK). Only CD34<sup>+</sup> events with low side scatter were counted as CD34<sup>+</sup> cells. CD34<sup>+</sup> cells were cryo-preserved in liquid nitrogen until use in long-term culture assay.

#### ***Long-term culture of CD34<sup>+</sup> cells on MSC derived stromal layer***

MSC cultures, at second passage, were plated into 25cm<sup>2</sup> vented flasks at 7.5x10<sup>4</sup> cells per flask (seeding density = 3000 cells/cm<sup>2</sup>) in 5ml of long-term culture medium (LTCM) (IMDM (Sigma-Aldrich, Gillingham, UK) containing 10% FCS (StemCell Technologies, London, UK), 10% horse serum (StemCell Technologies, London, UK), hydrocortisone (5x10<sup>-7</sup>M) and 1% Penicillin/Streptomycin. Cells were fed every week with LTCM by half medium exchange. After three weeks long-term culture cells were treated with chemotherapeutic agents for 48hours. Cultures were then washed repeatedly to remove all traces of the chemotherapeutic agents and all experiments were seeded with identical cryo-preserved CD34<sup>+</sup> cell populations derived from the same cord blood source (2.5 x 10<sup>4</sup> cells/flask (seeding density = 1000 cells/cm<sup>2</sup>)) in LTCM. Flasks were incubated at 37°C and fed weekly with LTCM by half media exchanges. All media removed each week when feeding was

assessed for the numbers of supernatant cells present and CFU-GM content using the CFU-GM assay described below.

CD34<sup>+</sup> cells are unable to produce stroma under the culture conditions used so it can be assumed that stromal elements grown in culture were MSC-derived [34]. As flow cytometric analysis indicated that there was no significant contamination of MSC cultures with cells of hematopoietic origin, it was decided not to irradiate MSC cultures prior to CD34<sup>+</sup> cell seeding as it was hypothesized that MSCs may have an altered sensitivity to radiation and DNA damage post chemotherapy exposure.

#### ***CFU-GM assay***

Supernatant cells removed from long-term cultures each week were plated at a concentration of 10<sup>4</sup> cells/well (seeding density = 26316 cells/cm<sup>2</sup>) into 0.25ml of MethoCult® GF H4434 (StemCell Technologies, London, UK) in triplicate into 12-well tissue culture plates. The number of hematopoietic colonies, which are derived from the CB CD34<sup>+</sup> cell population, present within each well after two weeks culture at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> was then assessed.

#### ***Human Umbilical Vein Endothelial Cell (HUVEC) culture***

The HUVEC cell line was obtained from the European Collection of Cell Cultures (ECACC) and grown in 75cm<sup>2</sup> vented fibronectin coated culture flasks. Cells were seeded in fibronectin coated flasks at a concentration of 1 x 10<sup>4</sup> cells per cm<sup>2</sup> in Endothelial Cell Growth Medium (ECACC) and fed by half medium exchange every other day and trypsinised every week.

#### ***CD34<sup>+</sup> cell transmigration assay***

For transmigration experiments 1x10<sup>4</sup> HUVEC cells were seeded on human fibronectin coated 5µm Millicell-polyethylene terephthalate membrane hanging transwell inserts for 24-well plates (Milipore®). Inserts were prior incubated with 0.01mg of human fibronectin (Millipore) in 0.1ml of DMEM for approximately 1 hour. After three days, the monolayers had reached confluence and were suitable for use within the assay.

MSC cultures in 24-well plates were treated with cyclophosphamide or melphalan and incubated for 48 hours. Cultures were washed repeatedly to remove all traces of the chemotherapeutic agents and suspended in 600µl of

MSC media. The Millicell transwell membrane inserts containing a HUVEC monolayer attached to the upper membrane surface were added to the 24-well plates containing the MSC cultures. A total of  $6 \times 10^4$  CD34<sup>+</sup> cells in 400µl of MSC media were added into the upper chamber of each transwell insert placed in the 24-well plate. The transwell set-up thus consisted of an upper and lower chamber separated by an endothelial cell layer. After 24 hours, non-adherent CD34<sup>+</sup> cells that had transmigrated the endothelial cell layer into the lower chamber were recovered and enumerated using a progenitor cell (CFU-GM) assay, as described above.

#### ***Cell cryopreservation and thawing***

Cell counts were obtained and cell density adjusted to  $<1 \times 10^7$  cells/ml with DMEM supplemented with 20% FCS, to which an equal volume of DMEM/20% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) was added. The vials were cooled until they had reached  $-80^\circ\text{C}$  using a 5100 Cryo 1°C freezing container (Nalgene, DK). Tubes were then transferred to a liquid nitrogen container for permanent storage until use.

Vials containing cells were thawed in a  $37^\circ\text{C}$  water bath with constant agitation. Cells were washed with DMEM/20% Foetal calf serum (FCS), centrifuged, and a cell count taken using Trypan blue exclusion (Sigma-Aldrich, UK) to determine live cell numbers. The cells were then resuspended in an appropriate pre-warmed medium for use.

#### ***Statistics***

All results within this study were expressed as the means +/- one standard error. Statistical comparisons were made by the paired t-test, repeated measures ANOVA, or 2-way ANOVA with Bonferroni corrections where appropriate. A value of less than  $p < 0.05$  was considered as significant.

## **Results**

### ***MSC characterization***

Cells harvested from femoral shaft marrows displayed all the typical characteristics of MSC in culture. At 3<sup>rd</sup> passage MSCs were uniformly positive for the mesenchymal markers CD105, CD166, CD44, CD29 but negative for CD45, which is consistent with the known MSC phenotype and excludes contamination of cultures with hematopoietic cells [26] (figure 1A). Mesenchymal stem cells were induced into adipogenic, osteoblastic and chondrogenic differentiation by culturing MSC, at third passage, in NH Adipodiff medium, NH Osteodiff medium and NH Chondrodif medium (Miltenyi Biotec, UK) respectively according to the manufacturers' instructions. Adipogenic, osteogenic and chondrogenic differentiation were characterised using the methods described (figure 1B).

### ***Morphology of MSCs after exposure to chemotherapeutic agents***

Upon treatment (48h) with cyclophosphamide, or melphalan, significant morphological abnormalities were noted within several treated cultures using phase contrast microscopy (figure 2A). Untreated cultures were morphologically homogenous and characterized by their fibroblast-like appearance with a highly uniform pattern of growth. Cells treated with cyclophosphamide displayed a highly diverse pattern of growth and granular appearance within their cytoplasm. Although having a slightly abnormal appearance, MSC cultures treated with melphalan displayed lower levels of visual morphological changes.

### ***Cytotoxicity assay***

Chemotherapy induced MSC cytotoxicity was determined using a colorimetric method for determining the number of viable cells in culture after treatment. Cell viability was calculated by measuring the absorbent value at 490nm based on a significant positive linear correlation between the optical density of the culture media at 490nm and MSC numbers present (data not shown). When examining the chemosensitivity of MSC cultures, the exposure to cyclophosphamide or melphalan did not result in any significant changes in the number of viable cells after treatment for 48 hours at biologically relevant concentrations ( $p>0.1$ ) (figure 2B).

### ***Expansion capacity of treated MSC cultures***

To examine the *in-vitro* expansion capacity of MSCs treated *in-vitro* with cyclophosphamide or melphalan, cells were treated and re-plated every 14 days at  $7.5 \times 10^4$  cells per flask and the total number of cells harvested at the end of each passage was found to calculate the cell population doubling rate (figure 2C). The expansion of untreated marrow MSCs, n=6, was 6.74 population doublings (SE=0.24) after 5<sup>th</sup> passage. The expansion of treated MSCs was lower at 5<sup>th</sup> passage, with a population doubling of 6.01 for cyclophosphamide (SE=0.52 n=6), and 0.40 population doublings for melphalan (SE=0.40 n=6). Differences in the expansion of untreated and melphalan treated MSCs were statistically significant ( $p < 0.01$ ). No significant differences in expansion were evident when comparing untreated and cyclophosphamide treated MSCs ( $p > 0.05$ ) (figure 2C).

#### ***CD44 in-vitro drug sensitivity measurements to chemotherapeutic agents***

MSC cultures were treated with chemotherapeutic agents and evaluated for the cell-surface expression of CD44 using flow cytometry, calculated using mean fluorescence intensity (MFI) values. Following incubation for 48 hours with different chemotherapeutic agents, MSCs demonstrated a significant reduction in the CD44 cell membrane surface expression after treatment with cyclophosphamide ( $p < 0.01$ ) (figure 3A). A maximal reduction of 28% in CD44 levels was seen after treatment with cyclophosphamide. No significant changes in MSC CD44 expression were evident after treatment with melphalan ( $p > 0.28$ ) (figure 3A). To examine the long-term effects of chemotherapeutic treatment on CD44 expression by MSC, the levels of CD44 after cyclophosphamide were determined over an 8 week period. Decreased levels of CD44 expression on MSC were evident for up to eight weeks post *in-vitro* treatment with cyclophosphamide ( $p < 0.05$ ) (figure 3B), with no significant recovery in the level of CD44 expression for the entire culture period post treatment. After eight weeks MSC cultures were terminated due to cell monolayers lifting from the culture flask surface.

#### ***Immunoblotting for MSC CD44***

Western blot analysis was used to show the expression of CD44 on MSCs, and to confirm its reduction in expression after treatment with cyclophosphamide. CD44 band intensity was measured using ImageQuant™ Version 5.2 software (Molecular Dynamics) and used as a quantitative indicator of protein expression. Detection using a BRIC235 antibody revealed a CD44 protein band at approximately 37kDa for both untreated and treated cell lysates which corresponds to the expected weight of CD44s [35-39] (figure 3C). The CD44 protein band detected by western blot had an estimated weight of 37kDa, which corresponds to the molecular weight of CD44s structure predicted by its amino acid sequence [39]. Image analysis using ImageQuant™ software

calculated that cyclophosphamide resulted in a significant decrease ( $p < 0.01$ ) in expression of the 37kDa band, with an approximate 22% decrease in CD44 expression. GAPDH expression was used to ensure equal loading of cell lysates.

#### ***CD34<sup>+</sup> cell transmigration assay***

The ability of untreated and cyclophosphamide or melphalan treated MSCs to provide chemotactic signals to hematopoietic progenitor cells and induce transmigration across an endothelial cell barrier were investigated using a HSC migration assay. MSCs cultured within a lower chamber were treated with chemotherapeutic agents, and after 48 hours  $6 \times 10^4$  CD34<sup>+</sup> cells were added to the upper chamber of the transmigration system, with the upper and lower chambers being separated by an endothelial cell layer. The number of hematopoietic progenitor cells that transmigrated through the endothelial barrier of the upper chamber (figure 4A) into the lower chamber after 24 hours was then calculated using a CFU-GM assay (figure 4B). The number of CFU that transmigrated into the lower chamber containing untreated MSCs was 148 (SE = 6). In contrast the numbers of CFU that transmigrated into the lower chamber towards treated MSCs were 106 (SE = 3) (cyclophosphamide), 121 (SE = 6) (melphalan), and 63 (SE = 7) (no MSC). The presence of untreated or treated MSCs within the lower chamber resulted in significantly higher levels of CFU endothelial transmigration into the lower chamber when compared to no MSCs being present ( $p < 0.01$ ). The number of CFU that transmigrated into the lower chamber towards cyclophosphamide or melphalan treated MSCs was significantly lower than the numbers of CFU that transmigrated into the lower chamber towards untreated MSCs ( $p < 0.05$ ). To ensure CD34<sup>+</sup> cells were not adhering to the MSC and therefore not being removed for inclusion within the CFU-GM assay, MSC cultures within the lower chamber were trypsinized and put into CFU-GM assay conditions. The presence of CFU-GM were absent from the adherent cell populations obtained (data not shown).

#### ***Hematopoietic colony-forming assay***

The ability of MSC-derived stromal cells to support hematopoiesis was compared post cyclophosphamide or melphalan treatment in long term hematopoietic cell cultures. MSCs from all groups in long-term culture conditions developed the same microscopic appearance as stroma derived from fresh marrow buffy coat samples as reported by Wexler et al [25]. All adherent MSC-derived stromal cultures were primarily seeded with hematopoietic CD34<sup>+</sup> cells derived from the same cord blood source. Co-culture of MSCs with allogeneic cord blood CD34<sup>+</sup> hematopoietic progenitor cells resulted in the formation of cobblestone areas representative

of hematopoietic progenitor cell proliferation and differentiation (figure 5). Assessing hematopoietic activity, using a 2-way ANOVA with Bonferroni corrections, tests indicated that over the 6 week culture period there was a significant reduction in the numbers of CFU-GM present for up to 3 weeks in cultures seeded onto cyclophosphamide and melphalan treated MSC derived stromal cultures, when compared to the numbers of CFU-GM supported by untreated MSC derived stromal cultures, ( $p < 0.05$ ), (figure 5).

## Discussion

Chemotherapy and stem cell transplantation are well established treatments of hematological disorders and other cancers. The adverse effects of the chemotherapeutic agents used in these treatments on HSCs are well known, however the effects on the MSCs residing in the bone marrow are less well characterized. Studies have demonstrated that a recipient's stromal cells are damaged after bone marrow transplantation [6-8], or even from chemotherapeutic drugs alone [6-15]. We have recently demonstrated a significant reduction in MSC expansion and MSC CD44 expression by MSCs derived from patients receiving HDC regimens for hematological malignancies [16]. Examining the effects of chemotherapeutic agents on patient MSC *in vivo* is difficult due to variability in patients and differences in the drug combinations used, both of which could have implications on MSC function. With drugs not being commonly used as single agents during HDC regimens there is a lack of data comparing the short or long term effects these drugs have on patients post treatment. To address these problems the effects of the commonly used alkylating chemotherapeutic agents cyclophosphamide and melphalan on human bone marrow MSC were evaluated *in-vitro*.

Both cyclophosphamide and melphalan belong to a class of alkylating agents known as nitrogen mustard derivatives. They are widely used in high-dose chemotherapeutic treatment of malignant disorders and in the preparative bone-marrow conditioning regimens pre-stem cell transplant. These compounds function as chemotherapeutic agents by the formation of DNA alkyl adducts and inter-strand DNA-cross links, inducing cellular apoptosis [40]. Knowledge of the clinical pharmacokinetics and pharmacodynamics of these agents administered in high doses is critical for the safe and efficient use of these regimens [41]. The concentration of both cyclophosphamide or melphalan within the bone marrow of patients undergoing high dose chemotherapy will be variable even after doses are adjusted for body surface area or weight, as there is considerable pharmacokinetic variability among patients receiving chemotherapy [42]. The drug concentrations used to treat MSCs in this study were therefore based on the mean peak concentrations of either cyclophosphamide or melphalan observed in the plasma of patients undergoing high-dose chemotherapy treatments [27-31].

Primarily the sensitivity of bone marrow MSCs to cyclophosphamide or melphalan induced cell death was investigated. Results indicated that exposure to these drugs did not cause any significant level of cell death to MSC cultures after 48 hours at the drug concentrations used. This resistance to apoptosis by cyclophosphamide is comparable to results seen by Li et al at identical drug concentrations [28], and may partially explain why



MSCs can be harvested from bone-marrow derived from patients who have received prior high dose chemotherapy [16, 43]. Although no significant loss of cell viability was observed, when investigating the expansion potential of MSCs post treatment, the mean expansion of untreated MSCs was over five times that seen of MSCs treated with melphalan after five passages. In contrast, cyclophosphamide treatment did not result in any reduction in MSC expansion. The cytotoxic effects of chemotherapeutic drugs, that result in DNA adducts, will result in alterations in gene expression and induction of mechanisms by which cells can detect DNA damage and control DNA repair. The DNA damage response, involving sensory and effector proteins, is strongly tied with cellular proliferation, cell cycle arrest, cellular senescence and apoptosis [42, 44]. Although investigating the exact mechanisms by which melphalan causes a decrease in MSC expansion would be desirable, it does demonstrate that chemotherapy disrupts the replicating ability of MSC, and corresponds with reductions in proliferative capacity of MSC harvested from patients who had received prior chemotherapy treatment [16]. This chemotherapy induced defect on proliferation is therefore a great concern as the ability/capacity for extensive proliferation and self-renewal is an essential property of a stem cell population. It could also explain why low numbers of mesenchymal progenitors are evident in patients after chemotherapy exposure [7, 11, 13].

MSCs have distinct structural properties from those of fully differentiated cells. Following exposure of MSC cultures to various chemotherapeutic agents structural abnormalities were evident with disruptions to the cell membrane morphology. MSC membrane surface expression of CD44 was also studied in response to the exposure of chemotherapeutic agents *in-vitro* as low CD44 levels seen on patients MSC after receiving high dose chemotherapy for hematological disorders [16]. CD44 is an adhesion molecule that plays a critical role in normal hematopoiesis [45-46], and it is the stromal microenvironment consisting of stromal cells and extra-cellular matrix that is thought to regulate and support the future fate of stem cells and committed progenitors along specific lineages [3-4, 47]. Using both flow cytometry and western blot techniques, the exposure of MSC cultures to treatment with cyclophosphamide resulted in a decrease in MSC CD44 expression, with a maximal decrease of 28%. Decreased levels of CD44 expression on MSC were evident for prolonged periods post treatment showing no sign of recovery.

The homing and engraftment of transplanted HSCs to the bone-marrow stem cell niche is essential for the establishment of intact hematopoiesis after high dose chemotherapy and/or total body irradiation in conjunction

with stem cell transplantation. It is thought that normal cell migratory processes involve events mediated through multiple adhesion/chemokine ligand interactions. Bone marrow cells are thought to secrete chemoattractants which facilitate the homing of circulating stem cells to the affected tissue to promote organ regeneration or tissue repair. The ability of MSCs to provide support for HSC migration after exposure to different chemotherapeutic drugs used in conditioning regimens is largely unknown. Intensive bone marrow transplant conditioning regimens, involving exposure to high concentrations of either cyclophosphamide or melphalan, create space within the marrow by removing the host's hematopoietic cells and also malignant cells in disease states in which the bone marrow is affected. In addition, chemotherapy agents used can also induce changes *in vivo* known to increase the homing potential of HSCs through disruption of the bone marrow endothelium barrier [48], and increased secretion of cytokines and chemokines which have impacts on HSC migration and repopulation [49-53].

The *in-vitro* transmigration model used in this study allows analysis of trans-endothelial migration of hematopoietic progenitor cells towards untreated and chemotherapeutically treated MSC layers. Homing is a fairly rapid process occurring no longer than 1-2 days [50], thus transmigration was observed during a period of 24 hours in these experiments. Firstly, it was demonstrated that the presence of MSCs (treated or untreated) within the lower chamber of the migration assay set-up resulted in an increased trans-endothelial migration of hematopoietic progenitors into the lower chamber when compared to no MSCs being present. This provided evidence that both treated and untreated MSC monolayers were able to produce chemo-attractive signals to assist in trans-endothelial passage and facilitate hematopoietic progenitor cell homing into the lower chamber. Subsequently it was found that the numbers of hematopoietic progenitors migrating through the endothelial layer, towards the MSCs within the lower chamber over a 24 hour period, were reduced after exposure of the MSCs to either cyclophosphamide or melphalan. As only MSCs were exposed to the chemotherapeutic drugs, the reduction in the rate of hematopoietic cell migration must result from a change in the secretion of a single or variety of soluble factors by MSCs post chemotherapeutic insult. These changes in the expression of soluble factors reducing HSC migration are either acting indirectly on the endothelial cells effecting trans-endothelial passage, or acting directly on the HSCs affecting their migration potential. Previous data has shown that there is increased homing of transplanted progenitor cells in non-irradiated non-ablative mice compared to TBI ablative preconditioned mice [54-55]. This inhibitory effect on stem cell homing caused chemotherapy exposure to MSC could in part explain the decrease in stem cell homing after ablative conditioning regimens.

Engraftment leading to hematopoietic recovery is vital following intensive chemotherapy/radiotherapy preparative regimens and stem cell transplantation. This engraftment is initiated by differentiating hematopoietic progenitor cells supported by the bone-marrow microenvironment. Engraftment may be slow or even fail following transplantation leading to prolonged pancytopenia or even death. This can happen despite an intensive preparative regimen, an adequate stem cell dose and complete donor chimerism [56]. It has been suggested that this lack of engraftment is due to functional damage caused by chemotherapeutic agents to the marrow microenvironment resulting in an inability to support the expansion of infused HSCs [7, 9]. Whether chemotherapy exposure affects MSC ability to form functional stroma and/or support hematopoiesis within the bone-marrow and has a role in changing hematopoietic engraftment kinetics in transplant patients is unknown. In long-term hematopoietic cell cultures the CFU-GM assay is used as an indirect measure of the number of primitive hematopoietic cells engrafting on MSC-derived stromal cells. Thus, the CFU-GM readout may be used to assess the ability of MSC-derived stromal cells to support hematopoiesis [57-58]. Data in this study demonstrate that MSC-derived stromal cells treated with either cyclophosphamide or melphalan display an abnormal stromal function in supporting early hematopoietic progenitor cells, with a reduced progenitor cell support for up to three weeks post treatment in long-term cultures. The role of MSC damage in engraftment kinetics is unknown; however it is known that MSCs can produce a number of early-acting cytokines which maintain HSCs in quiescence or promote their self-renewal, and also a variety of interleukins and cytokines which act on more mature hematopoietic progenitors [3-4]. Successful hematopoietic engraftment is heralded in 2-3 weeks by the appearance of donor progenitor cells in the circulation of the recipient [59]. The reduction in hematopoietic support by MSC-derived stroma observed within this time period, after cyclophosphamide or melphalan treatment, could therefore have large influences on patient engraftment and recovery.

## Conclusion

Disruptions in the ability of MSCs to undertake their normal biological function caused by cytotoxic damage during chemotherapy treatment could have huge consequences on hematopoiesis and tissue formation *in vivo*, as the multi-potential ability of MSC allows them to maintain host tissues during normal life and participate in tissue regeneration or repair in response to disease or injury [60]. Within this study the exposure of MSCs to the chemotherapeutic agents cyclophosphamide or melphalan had strong negative effects on MSC expansion and CD44 expression. In addition, changes were seen in the ability of MSCs to support hematopoietic cell migration and repopulation. Differences in cyclophosphamide or melphalan treatment on MSC function were evident. However, due to these drugs not being commonly used as single agents during high dose chemotherapy regimens there is a lack of data comparing the short or long term effects these drugs have on patients post treatment.

Collectively, these observations implicate the potential disadvantages in the use of autologous MSCs in chemotherapeutically pre-treated patients for future therapeutic strategies. Furthermore, this study lends to the hypothesis that if damage caused by chemotherapeutic agents to marrow MSCs is substantial, it would be logical to use cultured allogeneic MSCs therapeutically to assist or repair the marrow microenvironment after HDC.

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## Figures and Figure legends

Figure 1. (A) The phenotype of cells within MSC culture at 3rd passage (n=6). Results are expressed as the percentage of cells expressing the phenotypic cellular markers CD105, CD166, CD29, CD44 and CD45 in MSC culture (+/- SEM). (B) Images depicting MSC cultures at 3rd passage differentiated down the adipogenic, osteogenic and chondrogenic lineages. Adipogenic differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O (i). Osteogenic differentiation was visualized by the presence of calcium (ii). Chondrogenic differentiation was characterized by the immunofluorescent detection of aggrecan (green)/ nuclei (blue) (iii). Undifferentiated MSC cultures at 3<sup>rd</sup> passage show absence of staining with oil red O (iv), Alizarin red (v) and aggrecan (vi).

Figure 2. (A) Representative phase contrast images of MSC cultures treated with cyclophosphamide or melphalan. Cells treated with chemotherapeutic agents displayed a mixed morphology in culture with high levels of granularity present within their cytoplasm. (B) MSC viability after chemotherapeutic treatment. Results are expressed as a mean +/- SEM after treatment of MSC cultures with cyclophosphamide (n=6) or melphalan (n=6) (no significant differences in cell viability were evident between control and treated cultures,  $p > 0.05$ ). (C) Melphalan treatment damages the *in-vitro* long-term expansion capacity of MSCs. The graph depicts the expansion of untreated MSCs (n=6), or MSCs treated with the chemotherapeutics cyclophosphamide (n=6) or melphalan (n=6). Results are expressed as the mean +/- (SEM) (untreated vs melphalan  $p < 0.01$ ; untreated vs cyclophosphamide  $p > 0.05$ ; ANOVA).

Figure 3. Cyclophosphamide causes a significant reduction in MSC-CD44 expression. (A) The levels of CD44 expression, using MFI values, on MSCs exposed to cyclophosphamide or melphalan for 48 hours. Untreated (n=6), cyclophosphamide (n=6) and melphalan (n=6). (B) The levels of CD44 expression, using MFI values, on MSCs exposed to cyclophosphamide over an 8 week period post exposure. Results are expressed as the mean MFI level (+/- SEM) (untreated vs cyclophosphamide;  $*p < 0.05$ ). (C) Immunoblotting of CD44 on MSC samples post exposure to cyclophosphamide for 48 hours (n=4). Upper panels correspond to CD44; lower panel corresponds to GAPDH which was used to ensure equal protein loading.

Figure 4. The chemotactic property of MSCs is decreased after exposure to chemotherapeutic agents. (A) Phase contrast images of human umbilical vein endothelial cells in culture prior to being used in the CD34<sup>+</sup> cell transmigration assay. Images taken at (i) low and (ii) high magnification. (B) The frequency of hematopoietic progenitor cells (CFU-GM) transmigrating an endothelial cell layer towards an untreated MSC layer (Untreated), a chemotherapeutically treated MSC layer (Cyclophosphamide or Melphalan) or no MSC layer (No MSC). The number of transmigrated cells was measured after 24 hours. Results are expressed as the mean +/- SEM (\*p<0.05 when compared with number of CFU-GM values in the untreated control group).

Figure 5. (A) MSC-derived stroma treated with melphalan or cyclophosphamide display an abnormal stromal function. The graph depicts the number of CFU-GM present in culture after seeding cord blood CD34<sup>+</sup> cells into co-culture with MSC-derived stromal long term cultures exposed to cyclophosphamide or melphalan (n =6). Results are expressed as the mean (+/- SEM). (untreated vs cyclophosphamide or melphalan p<0.05 (3wks); ANOVA). (B) A phase contrast photomicrograph showing confluent and adherent MSC-derived stromal cells (white arrows) supporting the growth and differentiation of non-adherent cord blood derived CD34<sup>+</sup> cells (black arrows) when seeded into long-term co-culture.

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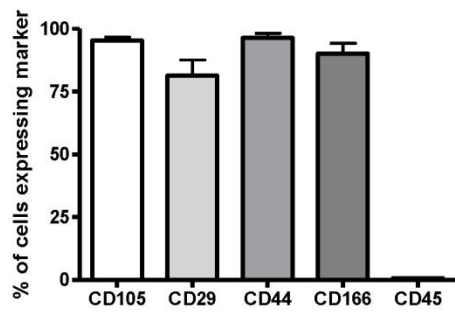
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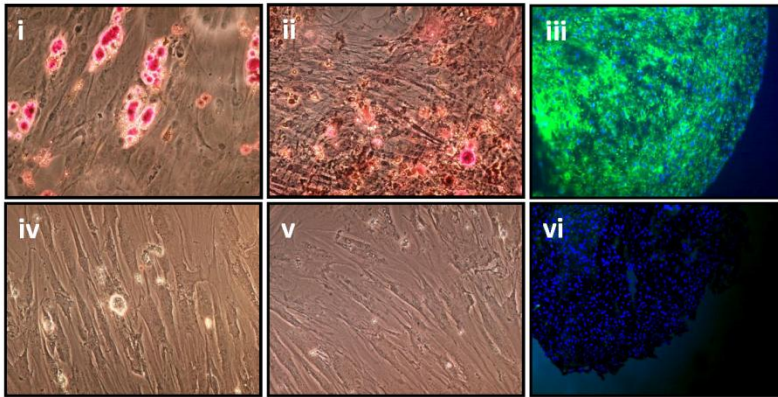
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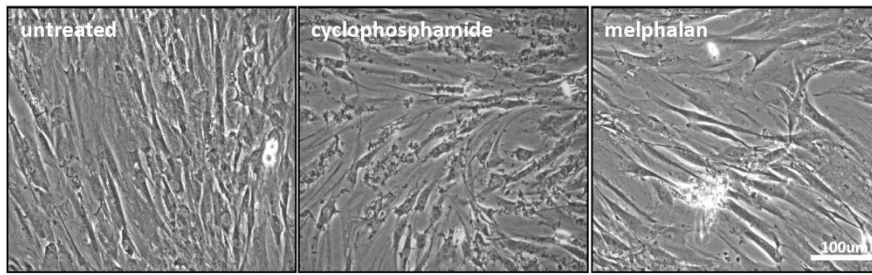
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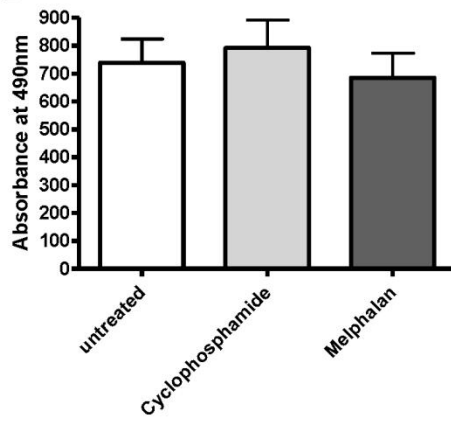
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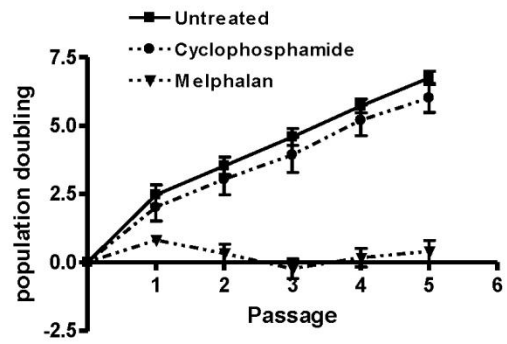
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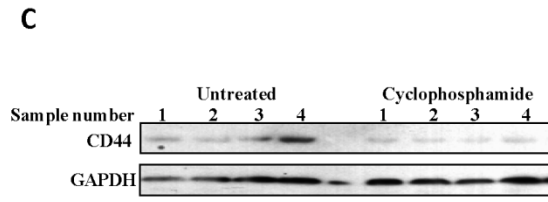
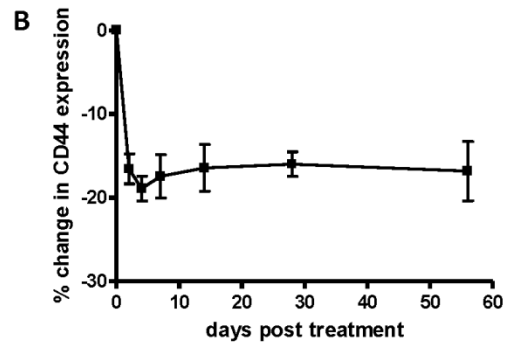
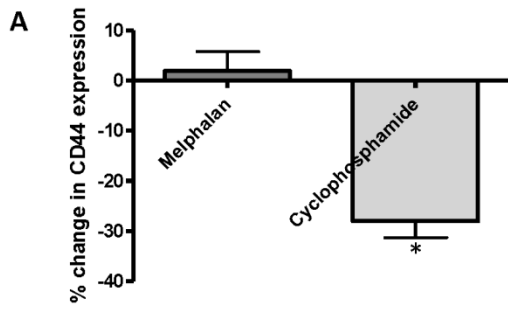


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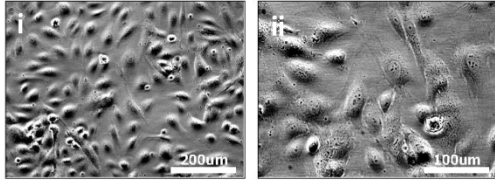
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