Stem Cells International

Clinical Perspectives of Mesenchymal Stem Cells

Guest Editors: Jan Kramer, Francesco Dazzi, Massimo Dominici, Peter Schlenke, and Wolfgang Wagner

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Editorial Board

Contents

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Editorial

Clinical Perspectives of Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) raise high hopes for regenerative medicine—in fact, they have already paved their way to a large number of clinical trials for a broad range of diseases. So far, several studies provided promising results, but this relatively new area of research requires further validation as some of the studies revealed varying outcomes. This might be a result of the heterogeneity of MSC cultures and absence of reliable protocols for isolation of the naïve stem cell fraction. MSCs are not precisely defined on a molecular level. They can be isolated from many tissues under different culture conditions—yet, they comprise multiple subpopulations and only a subset reveals multipotent differentiation capacity into at least adipogenic, chondrogenic, and osteogenic lineages. Notably, the composition of subpopulations seems to be greatly affected by culture methods and in the course of culture expansion.

Culture of MSCs has already been established in the early 1960s when fibroblastoid cells were discussed as supportive stromal cells within the hematopoietic bone marrow niche. Initially, application of MSCs in regenerative settings was mainly based on the hope to cure diseases or defects of cartilage, bone, or adipogenic tissue. Their use for musculoskeletal diseases still remains one of the most frequent applications (Figure 1). Particularly in an autologous setting, differentiated derivatives of MSCs may be functionally integrated in constructs to enhance regeneration of bone or cartilage defects.

There is a growing perception that MSCs reveal additional attributes which open further clinical perspectives: they seem to secrete active molecules which are capable to stimulate regeneration. The precise nature of these molecules, for example, growth factors, microvesicles, or direct cell-cell interaction, needs to be further specified. Yet, this stimulatory paracrine function may contribute to beneficial effects in applications such as ischemia, liver, and heart diseases. Furthermore, several studies have demonstrated that human MSCs reduce allorecognition, interfere with dendritic cell and T-cell function, and generate a local immunosuppressive microenvironment by secreting cytokines. This immunomodulatory function paved the way for cellular therapy in autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, or Crohns disease. Preliminary results with MSCs are promising for the treatment of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation.

Although we are only starting to understand the mechanism of repair, the ease of culture isolation of MSCs, their moderate side effects in ongoing trials, and their pleiotropic functions make them good candidates for cellular therapy. There is an urgent need for further randomized, double-blinded, placebo-controlled clinical trials to unequivocally demonstrate safety and efficacy of MSCs. These results will also feedback on basic research to optimize culture conditions and cell preparations for a given application. This

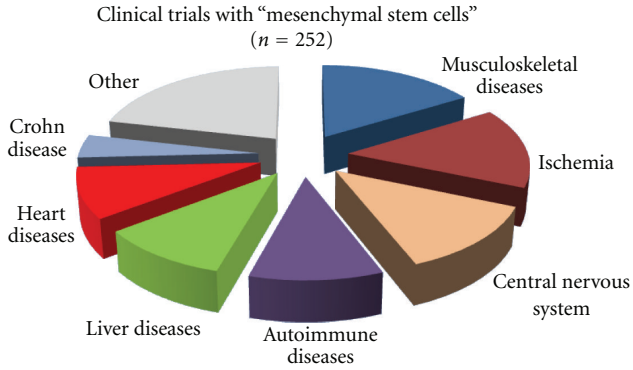

Figure 1: MSCs in clinical trials. 252 trials have been registered at http://www.clinicaltrials.gov/ (including 46 in North America, 61 in Europe, and 92 in East Asia, assessed on 11/20/2012). MSCs are tested for a broad range of diseases, and selected categories are presented.

special issue summarizes review papers and clinical trials to provide insight in clinical perspectives of MSCs.

Several methods for isolation of MSCs from human bone marrow (BM) have been described: they are commonly isolated from the mononuclear cell fraction upon density gradient centrifugation. Alternatively, MSCs can be isolated by direct plating of BM. K. Mareschi and coworkers have compared these isolation regimens in "*multipotent mesenchymal stromal stem cell expansion by plating whole bone marrow at a low cellular density: a more advantageous method for clinical use*". The results demonstrate that plating of whole bone marrow provides a suitable alternative for isolation of MSCs with relatively little hematopoietic contamination and slightly longer telomeres at first passage. Furthermore, the authors have addressed the impact of seeding density. Overall, this study supports the notion that MSCs have a diverse repertoire of distinct subpopulations which need to be taken into account.

Alternatively, MSCs can also be isolated from adipose tissue (AT). These cells play a role in autologous lipotransfer for soft tissue reconstruction, and they can also be culture isolated. The anatomical location and the harvesting method may influence AT-MSCs and this has been addressed by M. Aguena and coworkers in "*Optimization of parameters for a more efficient use of adipose-derived stem cells in regenerative medicine therapies*": comparison of samples from the lower abdomen *versus* flank revealed a significantly higher number of nucleated cells and expression of MSC markers in samples from the abdomen. Comparison of either pump-assisted liposuction or manual lipoaspiration did not affect cell numbers. These results exemplify the crucial role of starting material and cell isolation methods.

BM-derived MSCs have been described now over decades with regard to their osteogenic capacity. In this issue E. Zomorodian and M. Eslaminejad give an actual overview about "*Mesenchymal stem cells as a potent cell source for bone regeneration*". First, the authors give a short summary about MSC from different tissues. But regardless of which source, osteogenic differentiation of MSC *in vitro* always has to be induced by inductive factors. Although many exogenous osteoinductive reagents have been described, sometimes the specific molecular pathways by which the cellular differentiation processes are modulated still need to be clarified. In addition, a better understanding of the *in vivo* migration of MSC to defect sites might improve their therapeutic use for bone repair strategies in the future. Another future prospect might be the application of MSC as vehicles for bone gene therapy, but also in this field many issues have to be solved.

M. Mazo and colleagues review in "*Mesenchymal stem cells and cardiovascular disease: a bench to bedside roadmap*" the promising actions of MSC on injured myocardium by paracrine activity as well as differentiation into cardiovascular cell lineages. This comprehensive review paper demonstrates advantages of cellular therapy. However, the authors also point out that there are still many open questions at the level of basic research and animal models as well as even more at the outcome of clinical studies.

N. Venkataramana and coworkers have demonstrated in "Bilateral transplantation of allogenic adult human bone marrow-derived mesenchymal stem cells into the subventricular zone of parkinson's disease: a pilot clinical study" the safety of the procedure in 12 patients one year after the intervention. The authors assume beneficial neuroprotective and neurorestorative effects of MSCs. However, mixed results were obtained in this study, and only some patients showed a clinical improvement. This might be due to the fact that the duration of the disease varied widely in the study group. In addition, profound differences in the observed cell properties were mentioned, although the MSCs were only isolated from three different donors. Again, this clarifies that more basic work has to be done to enable a better definition of MSC (sub-) populations for a stable and reliable transplantation procedure in clinical settings.

MSCs mediate immunomodulatory effects. It might be possible that not only one subset of naïve stem cells but almost all mesenchymal stromal cells exhibit this immunogenic capacity. For example it has been demonstrated that mesenchymal stromal cells in general inhibit T-cell function. The development of GVHD is caused by T-cell reactivity. Kuzmina and coworkers contribute to this issue with the paper "*Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease: a phase ii study*". In this clinical study 19 patients received the standard GVHD prophylaxis with immunosuppresive in combination with the infusion of the MSCs of the hematopoietic stem cell donor during leucocyte recovery by activation of the hematopoietic transplant. This group was compared to 19 patients who were treated with the GVHD standard prophylaxis alone. In the MSC group only one patient developed acute GVHD, while in the standard group 6 patients suffered from this life-threatening disease. No differences in the graft rejection rates or in the incidence of infections were observed in both groups. But the overall mortality was 22.2% in the standard prophylaxis group compared to 5.3% in the MSC-treated group.

Taken together, MSCs provide promising perspectives for clinical applications with enormous potential for development, but the definite areas of application need to be

further specified and validated. At the same time, a better molecular understanding is required for quality control and standardization of cellular therapeutics.

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Clinical Study

Optimization of Parameters for a More Efficient Use of Adipose-Derived Stem Cells in Regenerative Medicine Therapies

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Adipose tissue-derived stem cells (ASCs) association to fat in autologous lipotransfer is promising for a more effective soft tissue reconstruction, and optimization of protocols to isolate ASCs from lipoaspirate fat is much needed. We demonstrated that an increase in adipocyte differentiation is dependent on the number of ASCs. In a sample of 10 donors, we found a higher concentration of nucleated cells in the lower abdomen compared to flank ($P = 0.015$). In a sample of 6 donors we did not find differences in the cell yield obtained by manual or pump-assisted aspiration ($P = 0.56$). We suggest that the increase in the number of ASCs in the reinjected fat may enhance the efficiency of newly formed adipose tissue and that the anatomical region from which to harvest fat tissue needs to be considered to optimize the number of ASCs in the harvested tissue. Finally, pump-assisted aspiration can be used without any significant harm to the viability of cells.

1. Introduction

Fat transfer has been used over the past decades as an autologous dynamic filler in plastic surgery rehabilitation in several circumstances, such as in reconstruction of damaged adipose tissue due to burn injury, for craniofacial reconstruction due to congenital defects or trauma, in cancer or other tumors as well as for aesthetic reasons. Although this approach is considered successful, the need of several surgical interventions to reach the aimed result is not unusual. Therefore, the development of alternative protocols to achieve more effective reconstruction of soft tissues is of major interest. Nowadays, a current promise is to enhance adipose tissue survivability by the combination of fat transplantation and stem cell therapy, particularly with the use of adipose tissue-derived stem cells (ASCs), since stem cells potentially ameliorate neovascularization and partially halt inflammatory response [1, 2].

Adipose tissue-derived stem cells (ASCs) can be easily isolated from the stromal-vascular fraction (SVF) of human adipose tissue by simple surgical procedure, can be obtained repeatedly, in large quantities and, in some cases, under local anesthesia, and are capable to undergo *in vitro* differentiation towards osteogenic, adipogenic, neurogenic, myogenic, and chondrogenic lineages when treated with specific factors [3, 4]. Multipotentiality of ASCs makes them interesting and promising candidates for mesodermal defect repair and disease management [5].

Regardless of using fat tissue as a sole fulfillment material or implemented with ASCs, there are several questions yet to be tackled in order to enhance its viability in tissue regeneration therapies. For instance, it is not well understood whether the percentage of ASCs in the reinjected fat tissue interferes in the efficiency of adipose tissue neogenesis. Moreover, the influence of the anatomical donor site in the number and type of mesenchymal cells is still uncertain: while some authors suggest that the subabdominal region is the most enriched site for mesenchymal stem cells (MSCs) [6, 7], others point out the hip as the best tissue to extract MSCs. Another important issue to be evaluated is the harvesting methodology, as some authors suggest that the

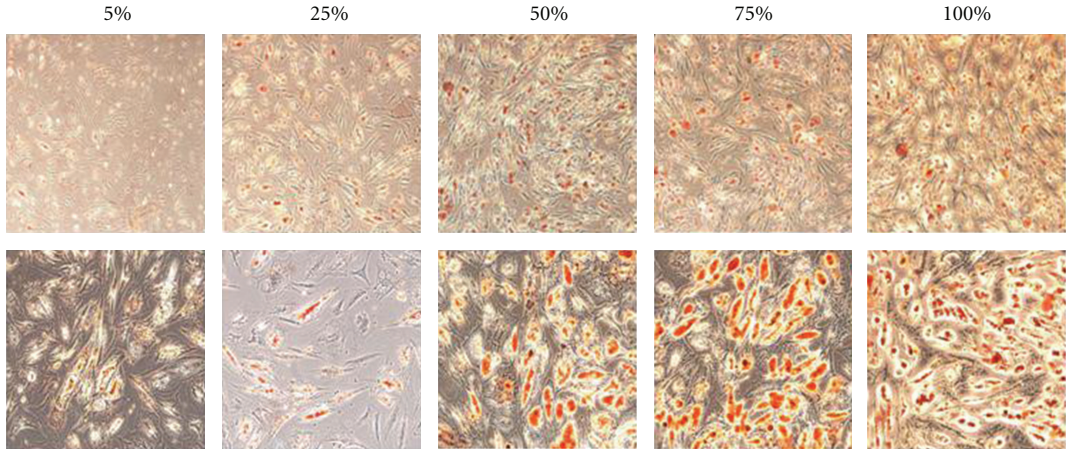

Figure 1: Oil Red-O staining for *in vitro* adipogenic differentiation of the mixed populations of ASCs and mature fibroblasts. Percentages indicate the proportion of ASCs. Pictures taken with 10X of magnification are showed in the upper line and pictures taken with 20X of magnification are showed in the lower line.

use of pump-assisted technologies decreases the viability of nucleated cells [8]. Considering that these factors might be controlled by the surgeon, their understanding will certainly have an impact in the final concentration of ASCs contained in the lipoaspirate [9].

In view of the questions listed above, the scopes of this work are to investigate if the proportion of ASCs influences the efficiency of the *in vitro* adipogenesis and to compare the quantity of nucleated cells in the SVF depending on the harvest area and finally, the harvesting methodology.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the Institute of Biosciences' Human Research Ethics Committee (permit number 095/2009-FR251136).

2.2. Lipoaspiration Surgeries. Lipoaspiration was done by a 10 mL syringe coupled to a cannula with a diameter of 2,5 mm, using the Coleman method [10] or by pump-assisted liposuction regulated at −350 mmHg using a cannula with a diameter of 3 mm. For pump versus manual lipoaspiration comparison experiments, 10 donors were submitted to sub-abdominal liposuction of right side with manual method and the sub-abdominal left side with pump method. For flank versus abdomen comparison experiments, 6 donors were submitted to liposuction in these distinct anatomical sites, with traditional pump suction lipoaspiration method. In order to minimize the differences between individuals, samples taken from the different locations and obtained using the different methods tested were paired in each series of subjects.

2.3. Adipose-Derived Stem Cells (ASCs) Isolation and Expansion. Adipose tissue from sub-abdominal and flank subcutaneous lipoaspirates were obtained by traditional pump suction from six healthy women undergoing cosmetic surgery procedures. In another instance, adipose tissue from sub-abdominal region of ten healthy women was isolated by either pump-assisted liposuction, with controlled negative pressure, or manual lipoaspiration. The adipose tissue were washed extensively with sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NA2HPO4, 2 mM KH2PO4 pH 7.4, reagents from Sigma-Aldrich) to remove contaminating debris and red blood cells. Washed adipose tissue was treated with 0.075% collagenase (type IA; Sigma-Aldrich) in PBS for 40 min at 37°C with gentle agitation) as described previously [4]. The collagenase was inactivated with 2 volumes of HBSS (Invitrogen, CA, USA) and the infranatant was centrifuged for 5 min at 3000 g. Concerning the samples used to test whether there is an influence of harvesting pressure on cell concentration, the cellular pellet for seven samples obtained by pump-assisted liposuction and seven matched samples obtained by manual lipoaspiration were resuspended in DMEM F12 (Invitrogen, CA, USA) supplemented with 15% FBS (Hyclone), 1% non essential aminoacids (Invitrogen, CA, USA), 1% penicillin-streptomicyn (Invitrogen, CA, USA) and seeded on conventional tissue culture flasks for immunophenotypical characterization.

2.4. Cell Count. Viable cells were counted using the trypan blue dye exclusion assay. A freshly prepared solution of 10uL trypan blue at 0.05% (Sigma Aldrich) in distilled water was mixed with 10uL of cellular suspension for 5 min., viable cells were counted in a Neubauer chamber using a light microscope (Nikon Eclipse TS100).

2.5. Immunophenotyping. Immunophenotype characterization of cell populations was done by flow cytometric analysis. For samples used in the comparison of cell yields depending on the anatomical region, we performed this characterization using the freshly isolated stromal-vascular fraction and each sample analyzed is a pool of the stromal-vascular fraction from five women donors. For samples used in the comparison of cell yields depending on the liposuction technique, the plated cell cultures were washed with PBS and digested

by trypsin solution (0,125% trypsin, 0,02%EDTA in PBS). The cells were incubated for 1 hour at 4°C with the following anti-human antibodies: CD29-PECy5, CD34PerCP, CD31-PE, CD45-FITC, CD90-R-PE, CD73-PE, CD105 (Becton, Dickinson and Company, NJ, EUA) and SH2, SH3, SH4 gently donated by professor Arnold Caplan (Case Western Reserve University). Matched control samples were incubated with PBS only. After a second wash with PBS, samples incubated with non-conjugated primary antibodies were incubated with anti-mouse-PE secondary antibody (Guava Technologies) for additional 15 min at 4°C. Cell suspensions were washed with PBS, fixed with 1% p-formaldehyde (Sigma-Aldrich) and 5.000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running the Guava Express Plus software (Guava Technologies Hayward, CA, USA).

2.6. Preparation of Heterogeneous ASC and Mature Fibroblast Culture. A coronal suture periosteal fibroblast cell lineage and an adipose derived stem cell lineage were grown to 80–90% confluence in independent flasks. The cell lineages were treated with 0,125% trypsin, 0,02% EDTA (Invitrogen, CA, USA) and the suspended cells were mixed in different concentrations of these two cell lineages, as following: 5% ASC and 95% fibroblast; 25% ASC and 75% fibroblast; 50% ASC and 50% fibroblast; 75% ASC and 25% fibroblast and 100% ASC only. Cells were plated in a concentration of $10^4/cm^2$ for each experiment. These mixed cell lineages were seeded in 12 well plates and after 24 hour, the mixed cells were induced to adipogenic differentiation.

2.7. In Vitro Adipogenic Differentiation. To induce adipocyte differentiation, cells were cultured in adipogenic induction medium DMEM high glucose supplemented with 10% FBS (Gibco-Invitrogen, CA, USA), 1% penicillin-streptomycin (Invitrogen, CA, USA), 1 μM dexamethasone (Sigma-Aldrich), 100 μM indomethacin (Sigma-Aldrich), 500 μM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/mL insulin (Sigma-Aldrich) for 14 days.

2.8. Oil Red O Staining and Quantification. For Oil Red O staining, cells were cultured in 12-well culture plates and, for each experiment; four wells per condition were used. After adipogenic differentiation for 14 days, cells were washed with PBS, fixed in 4% formaldehyde for 1 hour, and then cells were washed with deionized water. After, the cells were washed with 60% isopropanol and the cell plate was dried at room temperature. The cells were stained with 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 1 h at room temperature. Cells were then washed with deionized water three times to remove unbound dye and photographed. Stained Oil Red O was also eluted with 100% isopropanol (v/v) and quantified by measuring the optical absorbance at 500 nm. Oil Red O staining of undifferentiated cells grown in parallel culture served as the blank sample for this assay.

2.9. Statistical Analysis. Continuous variables were either expressed individually or by mean and standard deviation and we used the nonparametric Wilcoxon signed-rank test for paired data in order to assess if the population mean rank differ. To assess the statistical significance of the correlation between pump-assisted and manual liposuction and between the percentage of ASCs in mixed cellular populations and adipogenic differentiation we used two-tailed Pearson's correlation test. Tests with P values < 0.05 were considered to be statistically significant. All the applied tests were done by the use of the GraphPad Prism 5 program.

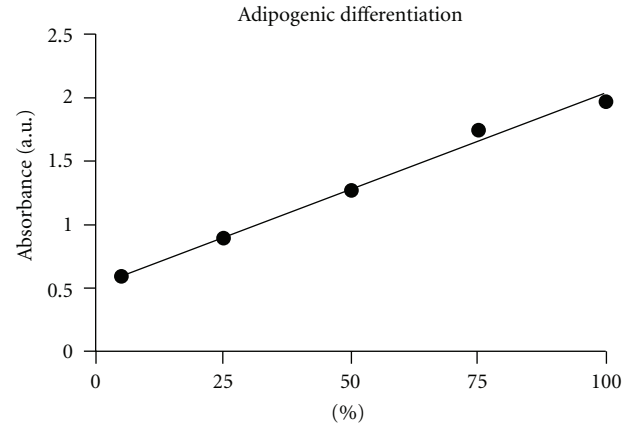

Figure 2: Linear regression showing significant correlation between the percentage of ASCs in the mixed cell population and the increase in Oil Red-O staining ($R^2 = 0.979$). Error bars represent standard deviation for quadruplicates of each sample.

3. Results

3.1. Influence of the Concentration of Adipose-Derived Stem Cells on the In Vitro Adipogenic Differentiation. To evaluate if there is an influence of the concentration of adipose-derived stem cells on the efficiency of the *in vitro* adipogenesis, we created five heterogeneous cell cultures composed of mixed subpopulations of ASCs and mature fibroblasts derived from cranial coronal suture, induced them to adipogenic differentiation and quantified the lipid vacuoles formation by Oil Red-O staining after 14 days (Figure 1). Applying a linear regression model, as showed in Figure 2, there is a significant correlation between the percentage of ASCs and the increase in Oil Red-O staining ($R^2 = 0.979$).

3.2. Influence of Donor Site on Cell Concentration. In order to determine if the number and proportion of mesenchymal cells varies according to the donor site of the adipose tissue, we evaluated cells from SVF from six female patients with mean age of 37 years (range, 26–51 years) submitted to liposuction at the lower abdomen and at the flank following medical recommendations. We observed a significantly higher concentration of nucleated cells in fat from the lower abdomen when compared to fat from the flank ($P = 0.015$; Figures 3(a) and 3(b)).

As showed in Figure 4, we observed a higher proportion of cells positive for mesenchymal (79.7–84.7%) and adhesion cell markers (CD29; 96%) in samples isolated from the abdomen as compared to the samples obtained from the flank (24.36–28.40% and 50.48%, resp.). Furthermore, samples

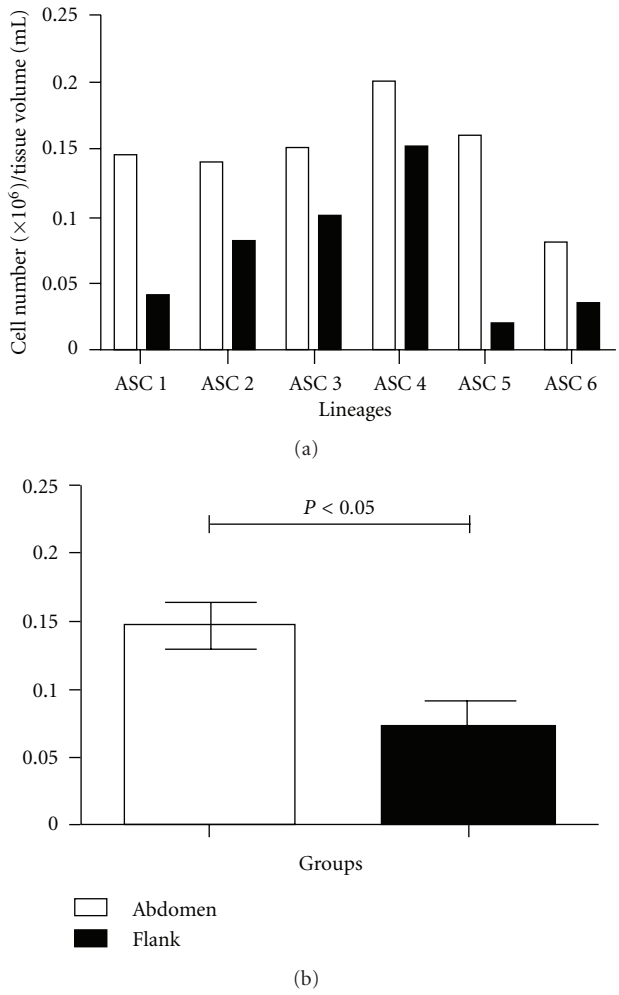

Figure 3: Bar graphs showing (a) cell quantity ($\times 10^6$) per volume of fat tissue (mL) obtained from abdomen or flank from six individuals and (b) Mean and standard deviation from data showed in graph "a". The Wilcoxon signed-rank test indicates significant difference between the samples ($P < 0.05$).

from the flank had a more prominent subpopulation of cells of hematopoietic (53.75%) and endothelial (21.56%) origin when compared to the ones from the abdomen (29.38 and 15.8%, resp.).

3.3. Influence of Harvesting Methodology on Cell Concentration after 1 Passage. A group formed by 10 patients, with mean age of 49 (range, 22–73 years) and mean body mass index of 24.88 (range, 22–29.6), submitted to sub-abdominal liposuction according to medical recommendation, was used to evaluate if there is an influence of the harvesting method on the yield of nucleated cells in the SVF. Liposuction was performed applying 2 commonly used harvesting methods: manual aspiration using a syringe and pump-assisted aspiration, with a controlled pressure of −350 mmHg. After 1 passage in culture, cells aspirated manually and obtained by pump-assisted aspiration showed positive staining (>89%) for mesenchymal (SH2, 3 and 4) and adhesion (CD29) markers and negative staining (<4%) for hematopoietic (CD45) and endothelial (CD31) markers. Fold change differences in the amount of cells obtained by pump-assisted method were calculated compared to manual aspiration and are represented in Figure 5(a). Applying a linear regression model (Figure 5(b)), there is no statistically significant difference on the cell yield obtained using these 2 methods.

4. Discussion

In autologous fat transplantation with large volume transfer, fat survival rather than fibrosis is desired. Other expected characteristics, which are usually highly variable, are the clinical longevity of fat graft and the maintenance of volume of the transplanted fat. To amend these variables, the combination of stem cell therapy with fat transfer, supplementing fat grafts with adipose-derived stem cells, has been reported as a method of autologous tissue transfer termed cell-assisted lipotransfer [9, 11, 12] and is a promise for the rehabilitation of several patients.

Using an *in vitro* model of admixtured heterogeneous cell populations we found a positive correlation between the percentage of ASCs and the increase in the *in vitro* adipocyte differentiation. Thus, we suggest that the increase in the number of ASCs in the reinjected fat tissue may enhance the efficiency of newly formed adipose tissue. These results would thus support the idea that enrichment of adipose tissue with mesenchymal stem cells can influence the rehabilitation process of patients submitted to autologous fat graft [5].

Therefore, establishing the ideal parameters to optimize the number of viable mesenchymal cells, such as settling preferred donor sites of lipoaspiration from which to isolate the ASCs and determining if the method of lipoaspiration interferes in the quantity and quality of the mesenchymal cells will certainly contribute to a more successful use of fat transplantation enriched with mesenchymal cells.

The finding of a significantly higher concentration ($P < 0.05$) of nucleated cells in the lower abdomen when compared to flank together with the observation of an enriched subpopulation of cells from mesenchymal origin in samples from the lower abdomen suggests that SVF from the lower abdomen might be a better source of mesenchymal stem cells than adipose tissue isolated from the flank. Our findings are consistent with the work of Padoin et al. [7], where they found a significantly higher concentration of nucleated cells in the SVF obtained from the lower abdomen when compared to upper abdomen, inner thigh, trochanteric region, knee, and flank. Jurgens et al. [6] also suggested, after CFU assays, that the abdomen is preferable to the hip/thigh region for harvesting mesenchymal stem cells.

If we take into account the number and type of mesenchymal cells, it thus seems that the adipose tissue from the lower abdomen is better than those from the flank. However, further studies are necessary in order to elucidate the functional effect on tissue regeneration as we do not know if the higher proportion of hematopoietic and endothelial cells contained in the flank might actually facilitate neovascularization, which is critical for the success of the surgery procedure.

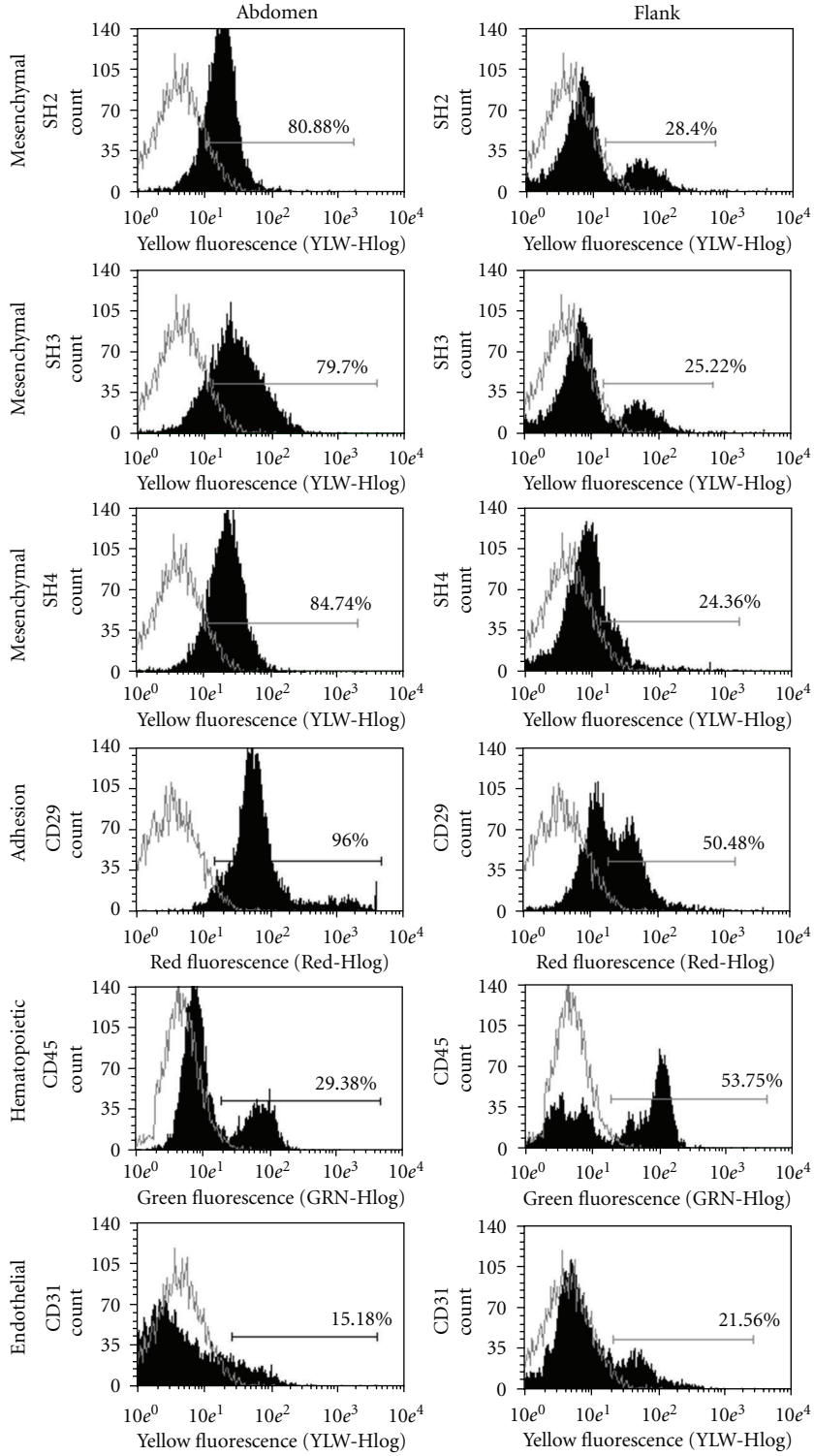

Figure 4: Immunophenotype characterization of fresh human stromal vascular fraction isolated from adipose tissue. The donors were submitted to liposuction in 2 different sites (abdomen and flank), and each sample analyzed is a pool of the stromal-vascular fraction from five women donors. Histogram for each sample (areas displayed in black) comparing cell number and fluorescence intensity with matched negative controls (areas displayed in grey). The percentage of positively stained cells is showed above each histogram.

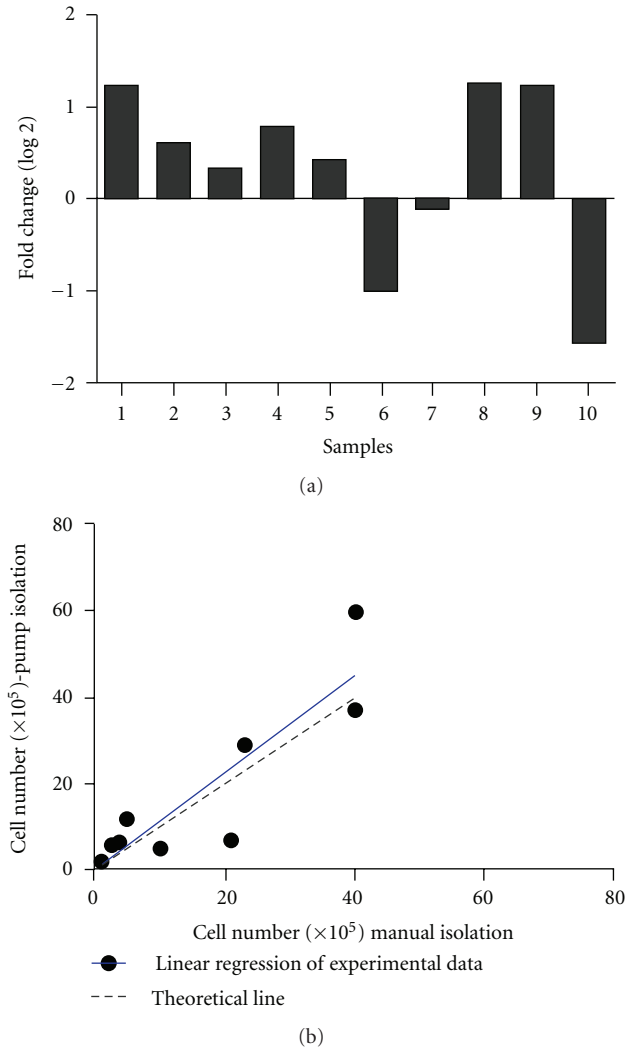

FIGURE 5: (a) bar graph depicting the $\log_2$ of the fold changes between the quantity of cells obtained by pump-assisted method compared to the amount of cells obtained by manual aspiration for ten individuals. (b) linear regression model showing no statistical difference in cell number depending on the liposuction method used. The difference in the slopes of the linear regression of experimental data (black line) is not significant ($P = 0.56$) compared to the theoretical line (dotted line, constructed assuming there is no difference between the data).

We did not find significant differences in SVF cell yields comparing manual liposuction using a syringe or pump-assisted lipoaspiration. Indeed, Fraser et al. [13] also tested these two approaches of fat tissue aspiration and concluded that the frequency of clonogenic cells was not impacted by the harvesting methodology. Although these data suggest no influence of these methodologies in the SVF nucleated cell quantity, a study by Mojallal et al. [8] concluded that liposuction at a controlled pressure of −350 mmHg gives a greater cell yield when compared to power-assisted aspiration with a negative pressure of 700 mmHg and syringe aspiration. However, these results should be considered with caution, as they performed this test using only 3 patients.

In this paper, we demonstrate that even though there is no difference on the nucleated cell yield obtained by manual aspiration using a syringe or with pump-assisted aspiration with a pressure of −350 mmHg, the anatomical region from which to harvest fat tissue needs to be considered as a means to optimize the total number of nucleated cells in the SVF and, consequently, the quantity of adipose-derived stem and progenitor cells. For the foreseeable future, implementations to the cell-assisted lipotransfer will lead to improve fat-grafting outcomes for restoration of tissues for either aesthetic or reconstructive purposes. Further, our results support the hypothesis that enrichment of adipose tissue with mesenchymal stem cells might improve the regeneration process following a cell-assisted lipotransfer.

Authors' Contributions

M. Aguena and R. D. Fanganiello contributed equally to this work.

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Clinical Study

Bilateral Transplantation of Allogenic Adult Human Bone Marrow-Derived Mesenchymal Stem Cells into the Subventricular Zone of Parkinson's Disease: A Pilot Clinical Study

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The progress of PD and its related disorders cannot be prevented with the medications available. In this study, we recruited 8 PD and 4 PD plus patients between 5 to 15 years after diagnosis. All patients received BM-MSCs bilaterally into the SVZ and were followed up for 12 months. PD patients after therapy reported a mean improvement of 17.92% during "on" and 31.21% during "off" period on the UPDRS scoring system. None of the patients increased their medication during the follow-up period. Subjectively, the patients reported clarity in speech, reduction in tremors, rigidity, and freezing attacks. The results correlated with the duration of the disease. Those patients transplanted in the early stages of the disease (less than 5 years) showed more improvement and no further disease progression than the later stages (11–15 years). However, the PD plus patients did not show any change in their clinical status after stem cell transplantation. This study demonstrates the safety of adult allogenic human BM-MSCs transplanted into the SVZ of the brain and its efficacy in early-stage PD patients.

1. Introduction

Shaking Palsy (*Paralysis Agitans*) or Parkinson's disease (PD) was originally described by James Parkinson in 1817 as "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured" [1].

Ever since the first description, scientists have pursued the causes and treatment of the disease. It is a chronic neurodegenerative disorder due to selective loss of dopaminergic neurons in the Substantia Nigra (SN) and the presence of proteinaceous inclusions known as Lewy bodies [2]. PD is recognized as one of the most common neurologic disorders, affecting approximately 15% of individuals older than 60 years. Between the ages of 75 and 84, that percentage may rise to almost 30%.

The causes of idiopathic Parkinson-disease (IPD) are believed to be a combination of genetic and environmental factors. Recent studies indicate that the pathogenesis includes a cascade of molecular and cellular events, oxidative stress, and release of reactive oxygen species (ROS), apoptosis, dysfunctioning of mitochondria and of the protein degrading system. Even immune-mediated mechanisms are being suggested for the progression and to explain the drug resistance that happens with time [2, 3].

The cardinal symptoms for PD are bradykinesia, rigidity, tremor, and instability which can be treated with dopamine replacement drugs. However, these drugs are unable to interrupt the progress of the disease and are ineffective against the disabling gait freezing, postural instability, lethargy, and lack of facial expressions. Also, over time there are drug-induced motor system complications; hence, it is suggested to delay the therapy till it significantly limits the patient's activities of daily living [4].

It is an established fact that much earlier to the clinical manifestations of the disease there are functional and structural changes in the nigrostriatal pathways which leads to a fall in the dopamine levels; releasing its inhibition and increasing the excitatory activities of the subthalamic nuclei and corticostriatal connections [1, 5]. Compensatory mechanisms are capable of maintaining the balanced neuronal output but not for long. Therefore, it was essential to search for alternate options to improve neuronal activity in the degenerated part of the brain. This lead to cell replacement therapies being identified as the most suitable option for PD as there is selective loss of dopaminergic neurons in the substantia nigra [6–10].

Over the years, numerous sources of dopamine-secreting cells like fetal mesencephalic tissue, human embryonic stem cells, and neural stem cells have been investigated with varying degrees of efficiency [6–8]. Although fetal mesencephalic tissue and embryonic stem cells showed a lot of promise, they are limited by the availability of fetal tissue and ethical concerns, persuading scientists to look deeper into the problem. This led to challenge the decade old hypothesis that regeneration is not possible in the brain. Stem cells were discovered in specific sites in the CNS such as the subventricular zone-SVZ (around the lateral ventricles) and their propensity to migrate to the traumatized areas of the nervous system. Numerous studies also confirm the differentiation capacity of stem cells into dopaminergic neurons in the presence of various external cues [11–13].

As a result, stem cells have emerged as a promising area helpful for tissue regeneration. Bone marrow mesenchymal stem cells (BM-MSCs) have the potential to differentiate into different lineages including functional dopaminergic neurons [14] without forming tumours. Animal studies illustrate that these cells have the property to migrate/home to the lesioned region as they respond to the chemoattractants released at the site [15–18].

An earlier report by our group in 2010 established the immediate and short-term safety of autologous bone-marrow derived mesenchymal stem cells in the transplantation therapy of PD and traumatic spinal cord injury patients [19]. Although the clinical improvement was only marginal, most of the patients experienced subjective well being without any notable side effects. Symptoms like freezing and facial expressions showed a tendency towards improvement. However, we noticed a variation in the BM-MSCs which were attributed to age and probably to long-standing disease. This encouraged us to carry out further studies in PD using BM-MSCs to overcome the variable in the previous study.

2. Materials and Methods

A clinical study was designed to determine the safety, feasibility, and efficacy of allogenic adult bone-marrow-derived mesenchymal stem cells in Parkinson's disease (PD) patients.

According to the national guidelines, Institutional Ethics Committee (IEC) approval was obtained for conducting the study. Necessary approvals for isolation, culturing, and transplantation of stem cells were also taken. Each patient who participated in the study was counseled on the procedure and informed consent obtained. The patient was screened for HIV, HBV, HCV, CMV, and VDRL by a nationally certified testing laboratory before being included in the study. All deviations to the protocol, drop outs, and adverse events were documented and informed to the IEC.

2.1. Study Design and Randomization. The study was conducted as a prospective, uncontrolled, one year, single centre safety, and efficacy clinical study of allogenic BM-MSCs bilaterally transplanted in patients diagnosed with PD.

2.2. Isolation of Mesenchymal Stem Cells from Healthy Adult Donors

2.2.1. Selection of Healthy Donors. Healthy donors were selected according to the donor inclusion criteria and as per the guideline of International Society of Cell Therapy (ISCT). Healthy donors were either male or female in the age group of 18–30 years of age, able to understand the voluntary donation program, and ready to provide voluntary written informed consent. Donors were excluded if they have illness such as autoimmune disorders, tuberculosis, malaria and any other infection, any illness which precludes the use of general anesthesia, history of malignancy, diabetes, hypertension, significant heart disease, genetic or chromosomal disorders, history of any inherited disorders, hemoglobin less than 10, and pregnant women.

At the time of obtaining informed consent they were screened for infection with human immunodeficiency virus (HIV), hepatitis B (HBV), hepatitis C (HCV), cytomegalovirus (CMV), and syphilis (VDRL) using reverse transcriptase-polymerase chain reaction (RT-PCR) method and excluded if found positive. They were also tested for complete blood count (CBC), renal function test (RFT), liver function tests (LFT), blood glucose, chest X-ray, Echocardiogram, and Electrocardiogram (ECG).

2.2.2. Isolation of Mesenchymal Stem Cells. BM-MSCs were isolated from healthy screened donors between the age of 18–30 years with informed consent. 60 milliliter of bone marrow was aspirated aseptically under local anesthesia from iliac crest of the healthy screened donors. The sample will be transported appropriately to the processing lab consisting of a class 1000 cGMP facility, and all sample processing was done in a class 100 biosafety cabinet. Bony spicules and particles were removed using a cell strainer and further diluted with DMEM-KO, centrifuged at 1800 rpm for 10 minutes at 20°C. The cells were resuspended with DMEM-KO and gently layered onto a density gradient solution (Lymphoprep, Axis Shield PoC AS, Oslo, Norway) to obtain an enriched mononuclear fraction. This was washed with DMEM-KO and centrifuged to collect the cells. The cells obtained were resuspended and plated in MSC complete culture medium consisting of Dulbecco's modified Eagle Medium (DMEM-KO) 10% fetal bovine serum (FBS) from preselected lots, and glutaMAX as described elsewhere [20]. The culture was maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 and subcultured prior to confluency.

2.3. Subculturing and Expansion. Once 80% confluent these cells were dissociated with 0.25% trypsin/0.53 mM EDTA (Invitrogen) and further upscaled and expanded in order to provide the required number of cells to the patient. Briefly, trypsinized cells were reseeded at a density of 1000 cells per cm^2 in cell stacks (Corning). After 14 days in culture, the cells reached 80% confluency and were ready for transplantation.

2.4. Quality Control Testing. MSCs were tested for quality control parameters such as Mycoplasma, Endotoxin, sterility and cell surface markers such as CD73, CD90, CD105, CD166, CD34, and CD45 markers using flow cytometry. They should be more than 80% positive for CD73, CD90, CD166, and CD105 but negative [<10%] for CD34 and CD45.

2.5. Characterization of Mesenchymal Stem Cells

2.5.1. Immunophenotype. Immunophenotyping of the cultured BM MSC was performed using flow cytometry to identify the presence of specific cell-surface antigens. Briefly, BM MSCs were dissociated with 0.25% trypsin-EDTA and resuspended in wash buffer at a concentration of 1×10 cells/mL. Cell viability was measured by flow cytometry using 7-amino actinomycin D (7-AAD) 200 μL cell suspension were incubated in the dark for 30 min at 4°C with saturating concentrations of phycoerythrin PE-conjugated antibodies. Appropriate isotype-matched controls were used to set the instrument parameters. After incubation, cells were washed three times with wash buffer and resuspended in 0.5 mL wash buffer for analysis. Flow cytometry was performed on a 5HT Guava instrument. Cells were identified by light scatter for 10000 gated events and analyzed. The following markers were analyzed: CD34-PE, CD45-PE, CD73-PE, CD105-PE, CD166-PE, and CD90-PE (BD Pharmingen, San Diego, CA, USA).

2.5.2. Differentiation. The trilineage differentiation capacity of human BM MSC into osteoblasts, adipo cytes and chondrocytes was investigated to confirm mesenchymal properties. Briefly osteoblast differentiation was induced by culturing human BM MSC in Stempro Osteogenesis Differentiation kit (Life Technologies, USA) for 15 days as per the recommendations provided by the manufacturers. Fresh medium was replenished every 3 days. Calcium accumulation was assessed by Von Kossa staining. The differentiated cells were washed with DP BS and fixed with 10% formalin for 30 min. The fixed cells were incubated with 5% silver nitrate for 60 min under ultraviolet (UV) light and then treated with 2.5% sodium thiosulphate for 5 min. Images were captured using an Nikon Eclipse 80i microscope (Nikon Corporation, Towa Optics, New Delhi, India).

To induce adipogenic differentiation, human BM MSC were cultured for 21 days using Adipogenesis differentiation kit (Life Technologies, USA) as per the protocol recommended by the manufacturers. Medium was replenished every 3 days. Cells were fixed in 10% formalin for 20 min, and 200 μL Oil Red O staining solution was added and incubated for 10 min at room temperature. The cells were rinsed five times with distilled water. The images were captured using a Nikon Eclipse 80i microscope (Nikon Corporation, Towa Optics, New Delhi, India). For chondrogenic differentiation, human BM MSC were cultured for 21 days using chondrogenesis differentiation kit (Life Technologies, USA) as per the manufacturer's recommendations and stained with Safranin O as specified. The images were captured using Nikon Eclipse 80i microscope (Nikon Corporation, Towa Optics, New Delhi, India).

2.5.3. Karyotyping. To rule out any chromosomal aberrations during *in vitro* propagation of BM MSC, these cells were karyotyped prior to transplantation. The chromosomes were visualized using a standard G-banding procedure, and more than 200 cells were analyzed per sample and reported according to the International System for Human Cytogenetic Nomenclature (ISCN).

2.6. In Process Test. Prior to dispatching the cells for transplantation, a battery of in-process quality testing was performed on the cells. These include morphology, immunophenotyping cell surface marker analysis, endotoxin testing using LAL test, and mycoplasma using RT-PCR was also done. Only those cells fulfilling the ISCT criteria for MSCs were released for transplantation.

Any sample positive for endotoxin and mycoplasma was discarded immediately and appropriately.

2.7. End Product Test. The final cell suspension which was provided to the clinician for transplantation was again tested for cell surface marker analysis as mentioned above. In addition, karyotyping, endotoxin, and mycoplasma were also performed as mandatory quality testing. Cell viability was measured by flow cytometry using 7AAD (7-amino actinomycin D). Certificate of analysis (COA) was prepared, and cells were released along with documentation for transplantation.

2.8. Patient Selection. Subjects, both male and female between 18–80 years, were enrolled for this study. The patients were screened for HIV, HBV, HCV, CMV, and VDRL followed by inclusion criteria selection before participating in the trial. 8 PD patients and 4 PD plus syndrome patients were chosen for the trial (those patients diagnosed with multiple system atropy and progressive supranuclear palsy (PSP) have been classified under PD plus syndrome patients). This would help us understand the role of bone-marrow-derived mesenchymal stem cells in the early stages of the disease and in rapidly progressing PD plus syndrome patients.

Those patients who fulfilled the following inclusion and exclusion criteria were included for the study.

Inclusion Criteria

(1) Should be in the age group of 18–80 years.

(2) Should be fully conscious, alert, and oriented while providing consent.

(3) Should show significant motor and nonmotor symptoms.

(4) Subject should provide a written informed consent and agree to return for follow up.

(5) Subject should be clinically diagnosed for Parkinson's disease and PD included disorders with motor complications despite adequate oral anti-Parkinsonian therapy.

(6) Should be able to comply with and understand the required visit schedule.

Exclusion Criteria

(1) Patient is suffering from Dementia (MMSE < 25).

(2) The extent or severity of the disease is not measurable.

(3) If the subject suffers form preexisting medical conditions such as bleeding disorders, and septicemia.

(4) Patients with a past (within one year) or present history of psychiatric disorder.

(5) If the subject has been enrolled in other investigational drug trial or has completed any trial within the last 3 months.

(6) If hemoglobin < 10 gm/dL, serum creatinine < 2 mg/dL, serum total bilirubin < 2 mg/dL, and HbA1c < 7%.

(7) Pregnant or nursing or women in child bearing age without adequate contraception.

(8) The subject tested positive for HIV, HCV, HBV, CMV, or VDRL.

2.9. Clinical Evaluation. These patients were admitted 48 hours prior to the procedure, and a detailed clinical evaluation was performed including UPDRS, MMSE, gait and neuropsychological assessments. General physical examination and cardiac status of the patients were evaluated before inclusion into the study. MRI was done at the baseline and at 12 month follow up. MR Tractography was conducted at 3rd, 6th and 9th month follow up sessions.

2.10. MRI Imaging and Tractography. MRI imaging was done on a 1.5 5T (Wipro GE, Milwaukee, WI, USA) machine. Routine imaging was done with axial FLAIR and T2W images and Sagittal T1W images. DTI of brain was done in the axial plane using an 8-channel CTL array spine coil with the following parameters-25 directions EPI tensor imaging (TR 8500, TE: 97.6 *b* value: 1000 frequency: 128, phase 128, NEX-1, slice thickness: 5 mm with zero interslice gap and bandwidth: 250 kHZ).

2.11. Image Processing. Image processing was done using FuncTool software provided by GE and quantitative analysis was done to calculate fractional anisotropy using standard methods. ROI were placed in bilateral centrum semiovale, genu, splenium of corpus callosum, anterior limb of internal capsule, posterior limb of internal capsule, and cerebral peduncles (total of 12 ROI).

2.12. Statistical Analysis. Descriptive statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean ± SD (min-max) and results on categorical measurements are presented in number (%). Significance is assessed at 5% level of significance. Student *t*-test (two tailed, dependent) has been used to find the significance of study parameters on continuous scale within each group.

2.13. Processing of Cells for Intracranial (IC) Transplantation. As mentioned in the earlier study [19] the cells were processed for transplantation. Briefly, after harvesting step, the total cell count was taken using a standard hemocytometer. The cells were washed several times with normal saline solution. Finally the cells were resuspended in saline containing 0.2% human serum albumin. The cell suspension (2 mL) was equally distributed into two 2 mL syringes and labeled. These were packaged in a sterile container and dispatched in a transportation container maintained at 22°C to the hospital for transplantation via the shortest route.

2.14. Surgical Procedure. The patient was positioned supine for the transplantation and the parts aseptically prepared. Under short propofol anesthesia bilateral frontal burr holes were drilled and small dural openings made. The sub ventricular zone was accessed through a standard brain cannula with CRW stereotactic frame or Stealth (Medtronic) navigation assistance. BM-MSCs, at a dose of 2 million cells/kg body weight, were transplanted into the brain and gelfoam placed over the dural defect prior to closing of the wound.

After operation the patients were observed in the neurointensive care unit for 24 hours following which they were shifted to the ward and discharged home on the 4th/5th day.

2.15. Evaluations and Follow-Up Schedule. The patients were followed up closely every three months for one year. They were assessed by an independent neurophysician and a movement disorders specialist. During each visit the patient was clinically examined, UPDRS score performed, neurologically assessed, and medications reviewed. At the final follow-up visit, that is, 12th month the patient would undergo a MRI scan to check for any structural changes in comparison to the baseline scan. The medication would be reviewed at each visit and adjusted based on the symptoms. Any adverse event would be reported to the concerned investigator and IEC.

3. Result

In this study, 12 patients were recruited according to the study design as per the inclusion and exclusion criteria mentioned above. This included 9 males and 3 females in the age group of 37–69 years. The duration of the disease varied between 3 and 15 years in the study group. Out of the 12 patients, 4 were diagnosed as PD plus and belonged to the older age group. The details of the patients who participated in this study are mentioned in Table 1.

2 million cells/kg body weight suspended in 2 mL of saline was implanted bilaterally into the subventricular zone

Table 1: Shows the details of the PD patients and PD plus patients recruited for the study. It also provides the subjective improvements reported by the patient/caregivers after stem cell transplantation. It is to be noted that all patients have mentioned improvement in similar aspects of PD including reduction in tremors, stiffness of limbs and clearing of speech. However, for PD, plus there are no improvements noted.

Sr. no.	Age/ sex	Duration of disease (years)-PD/PD+	Date of SCT	Type of stem cells injected	Dose administered	ROA	Autologous/ allogenic	No. of injections	UPDRS score baseline off/on	12th month off/on	Comments by caregivers/patient
1	68/M	15-PD	Feb-10	BMMSCs	2 million	IC	Allogenic	1	154/127	128/127	NA
2	37/M	5-PD	Mar-10	BMMSCs	2 million	IC	Allogenic	1	79/47	52/50	(1) Following stem cell injection his rigidity had reduced and speech had improved
3	65/M	5-PD	Mar-10	BMMSCs	2 million	IC	Allogenic	1	94/58	28/22	(1) Able to do his routine activities without much difficulty (2) Stiffness and tremors are very minimal (3) Able to walk for longer distances
4	49/M	10-PD	Dec-10	BMMSCs	2 million	IC	Allogenic	1	112/98	Lost to follow up	(1) Patient is more independent in his daily activities (2) Speech is clearer (3) Rigidity and stiffness has reduced
5	54/F	10-PD	Feb-11	BMMSCs	4 million	IC	Allogenic	1	105/72	90/68	After therapy the patient and relatives have noticed that there was a decrease in involuntary movements
6	48/M	11-PD	Feb-11	BMMSCs	2 million	IC + IV	Allogenic	1	43/32	40/28	NA
7	56/M	5-PD	Mar-11	BMMSCs	2 million	IC	Allogenic	1	NA	NA	NA
8	60/M	5-PD	Feb-11	BMMSCs	2 million		Allogenic	1	44/38	19/12	(1) After therapy, he has notice d that the tremors have reduced (2) Walking difficulty has also reduced and (3) He is able to write better now
9	65/F	10-PD +	Mar-10	BMMSCs	3 million	IC	Allogenic	1	44/20	NA	(1) Speed of walking has increased (2) Slurred speech has improved (3) Swallowing is better (4) Slight Improvement in handwriting
10	66/M	7-PD+	Jul-10	BMMSCs	2 million	IC	Allogenic	1	62/62	NA	According to the family, the symptoms have worsened since a few months
11	69/M	3-PD+	Jan-11	BMMSCs	2 million	IC + IV	Allogenic	1	NA	NA	(1) 6 months after therapy, the patient and his relatives have noticed improvement in his rigidity and movements (2) There is also improvement in his speech
12	69/F	7-PD+	4-Nov-10	BMMSCs	2 million	IC	Allogenic	1	150/153	140/133	(1) At present her involuntary movements have reduced (2) She has difficulty in swallowing and stiffness in her neck has increased

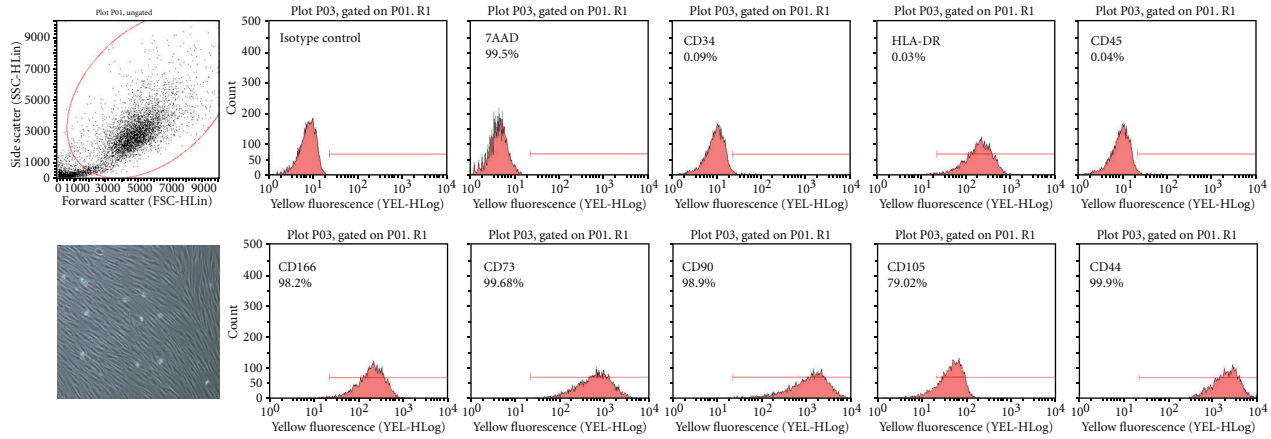


Figure 1: Characterization of adult BM-MSCs prior to transplantation as per ISCT criteria. It shows the plastic adhered spindle-shaped fibroblast like appearance of adult BM-MSCs in culture (lower left panel). And the surface expression of CD markers (top panel: negative markers and lower panel: positive markers).

using burr hole surgery technique. All patients tolerated the procedure well, there were no postoperative complications and were discharged within a week's time from the hospital. This indicates that there were no immediate cytotoxic effects due to implantation of allogenic bone marrow mesenchymal stem cells in to the sub-ventricular zone of the brain and the procedure was safe.

3.1. Allogenic BM-MSCs. 3 adult healthy screened donors were recruited for the aspiration of bone marrow under general anesthesia. BM-MSCs were isolated and cultured as described in [20]. These cells were cryopreserved and appropriately propagated once the patients were recruited for the study. All the cells were assessed for their morphology, immunophenotype and differential potential.

3.2. Characterization of BM-MSCs

3.2.1. Morphology and Immunophenotype. The cells displayed a typical spindle shaped fibroblast-like appearance as shown in Figure 1 and flow cytometric analysis shows the cell surface expression of CD markers (as per ISCT) as shown in Figure 1. The cells were found to be $CD34^-/CD45^-/CD73^+/CD90^+/CD105^+/CD166^+$ as depicted in Figure 1.

3.2.2. Multipotent Characteristics. In order to ensure that the BM-mesenchymal stem cells maintain their typical properties, the trilineage differentiation capacity of these cells was demonstrated. The cells were found to undergo adipogenic, osteogenic and chondrogenic differentiation as determined by Oil Red O stain, Von Kossa stain and Safranin O stains, respectively, (Supplementary data (Figure 2) will be available online at doi: 10.1155/2012/931902).

This set of analysis confirms that the cells being used for the clinical study are truly mesenchymal in nature.

3.2.3. Karyotype. All the samples used for transplantation were processed for karyotyping prior to transplantation by a trained cytogeneticist. No abnormalities/aberrations were noted after *ex vivo* propagation. A representative ideogram is shown in the Supplementary data (Figure 3).

3.3. Clinical Assessment. Clinical assessment was performed on all patients based on 4 basic parameters of the UPDRS scoring system: (1) mental behavior and mood, (2) activities of daily living, (3) motor disabilities and impairment, and (4) complications of PD therapy. This was considered as the primary measurable outcome of the clinical study. The scoring was typically done during the "off" period (approx.12 hours off the anti-Parkinsonian medication) and during the "on" period (within 1-2 hrs of the medication) where maximum benefit could be appreciated in the PD symptoms. The average score during the "on period" at baseline was 62.33 and after stem cell transplantation it improved to 51.16 that is, an improvement of 17.92% over the baseline (Figure 2). Similarly for the "off period", the average score was 86.5 at baseline and reduced to 59.5 after 12 months of stem cell transplantation. The percentage improvement in the "off period" score was 31.21% (Figure 3). This is similar to the data reported earlier by our group using autologous bone-marrow-derived mesenchymal stem cells [19].

Most of the PD patients reported subjective improvement during the first follow up that is, at 3 months after stem cell transplantation. These include clarity in speech, reduced tremors, and rigidity, and general sense of well being. These changes were seen in the later follow ups too indicating that the changes were not transient but more permanent. Similar improvements were also noted for some of PD plus patients but not all. However, for these patients the changes were transient and by the next follow up (6th month) most of them had progressed further into the disease.

3.4. Improvement in Relation to the Duration of the Disease. As depicted in Figure 5 and Table 2, there is a direct correlation observed between the duration of the disease and the improvements noted in the PD patients. Patients who had been diagnosed more recently performed better on the UPDRS compared to ones with the long-standing

Table 2: Shows the improvement in UPDRS scores for PD patients before and after stem cell transplantation.

Duration of PD (in yrs)	Percent of change in UPDRS scores	
	OFF period	ON period
5	34.17	6
5	70.21	62.06
5	56.81	68.42
10	14.28	5.55
11	6.9	12.5
15	16.12	1

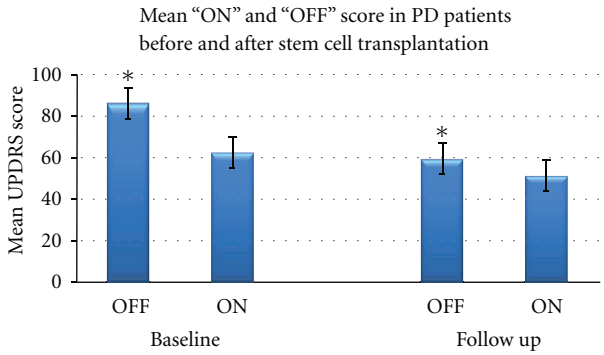


Figure 2: Shows the mean ± SD of the UPDRS score of the PD patients during "OFF" and "ON" periods at baseline and after stem cell transplantation. The scores have been assessed during the screening visit and final visit 12 months after transplantation. *Represents the level of significance.

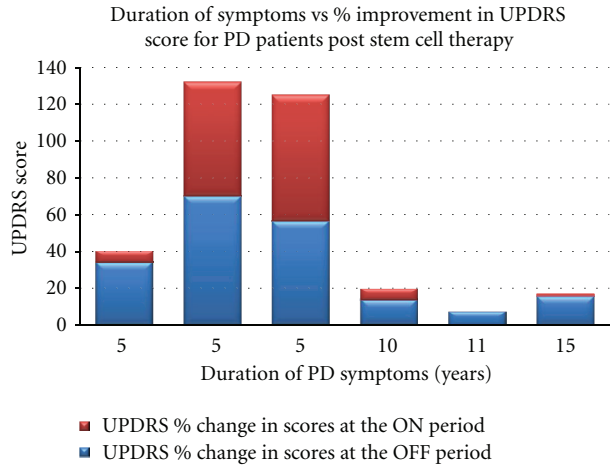


Figure 3: Illustrates the improvement in UPDRS scores for PD patients before and after stem cell transplantation. The graph highlights the fact that the patients treated early at the onset of the disease (5 years) have shown significantly better improvement which correlates clinically. The patients that were treated between 10–15 years after the diagnosis of PD did not show any significant improvement.

illness. Table 2 shows that those patients with PD for less than 5 years improved much quicker and remained in stable comparison to the patients suffering for more than 10–15 years. Whereas PD plus patients did not show improvement or such correlation after transplantation. Though there had been a subjective initial improvement, it never sustained in the long-term for PD plus syndromes.

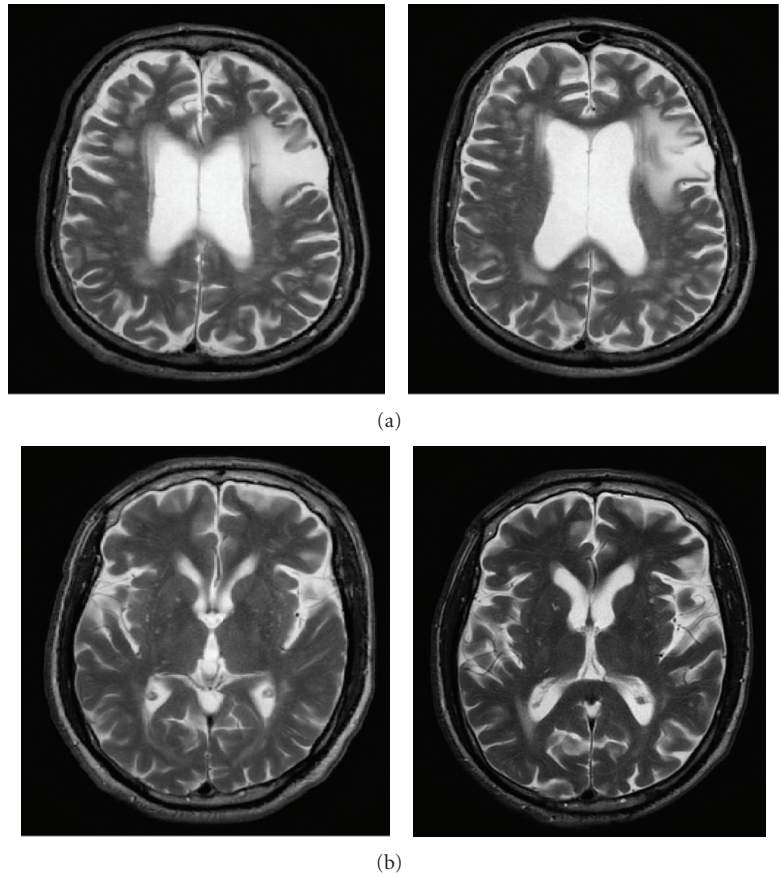
It needs to be mentioned that the PD plus patients could not be rated using the UPDRS scoring system after stem cell transplantation due to the severity and progression of the disease. Hence, this data has not been mentioned.

MRI Studies. MRI of the brain was done before and 12 months after stem cell therapy as shown in Figure 4. The brain images showed similar changes before and after treatment. No significant differences could be appreciated in the images. There were no structural changes, leukomalacia, or any additional growth observed. In one patient, incidental asymptomatic lacunar infarct was seen on follow up.

MR Tractography. The results of MR tractography have been shown in Tables 3(a) and 3(b). 12 different sites of the brain were analyzed during the different stages of follow up. A trend of improvement was observed in the genu and the peduncles steadily over a period of 12 months. The values improved from 0.53±0.13 to 0.69±0.07 in the right genu and 0.54 ± 0.14 to 0.63 ± 0.14 in the left genu and 0.50 ± 0.14 to 0.64 ± 0.04 in the left peduncle with a significant improvement on the 2nd follow up in the right peduncle. Interestingly in the PD plus patients, there was a further reduction in the values even after stem cell transplantation. It reduced from 0.378 ± 0.1255 to 0.3555 ± 0.1219 in the right genu and from 0.3875 ± 0.0723 to 0.3515 ± 0.1135 in the anterior limb of the internal capsule. 2 out of 4 patients have shown no improvement in FA values (i.e., FA values are decreasing in both the limbs of internal capsules on follow up scans). This correlated clinically with further deterioration of the symptoms in the PD plus syndromes.

3.5. Dose of Medication. The dosage of anti-Parkinsonism medication before and after stem cell transplantation was analyzed. For 4 PD plus patients, the clinician recommended an increase in dosage of medication based on their progression of the disease. However, for the PD patients in the arly stages of the disease similar increase in dosage was not required. The dosage has remained the same as the baseline medications prescribed. This indicates that the disease has not progressed further after stem cell transplantation. Only for 2 patients, the dosage of Syndopa had to be increased. This is probably because the disease had already advanced beyond repair at the time of stem cell transplantation which is also evident from the UPDRS scores of the patients.

Therefore, out of the 8 PD patients, intervention was in the early stages in 6 patients. The progression of the disease appears to have been slowed after the administration of stem cells. They did not require enhancement of dose. In the late stages of disease and PD plus patients, stem cell transplantation had shown relatively lesser symptomatic relief and on the other hand needed an increase in medications.



(a)

(b)

Figure 4: Shows T2 FLAIR axial images. (a) Depicts bilateral asymmetric multifocal hyperintensities involving pontine base periventricular and deep white matter suggestive of small-vessel ischemia. Diffuse brain atrophy is also seen with mineralization below globus pallidus and substantia nigra indicative of PD. There is no significant difference noted between the baseline and follow-up MRI. (b) Depicts moderate brain atrophy with bilateral putaminal rim sign seen. Small-vessel ischemic changes are seen in the bilateral periventricular and deep white matter. Mineralization of bilateral lentiform nuclei, dentate nuclei, and substantia nigra visualized is suggestive of MSA-P or PD plus syndrome. There was no significant difference noted between the baseline and follow-up MRI.

3.6. Activities of Daily Living. Care givers have noticed an overall improvement in their activity levels in 7 patients, which includes reduction in tremors both at rest and in motion, better clarity in speech, reduction in rigidity, ability to walk for longer distances and perform personal tasks independently. This has a significant impact on the well being of the patient and further substantiates the fact that the progression of the disease has been slowed down after stem cell therapy.

4. Discussion

The current treatment for PD includes pharmacotherapies and deep brain stimulation techniques. Lesioning surgery is gradually fading. However, these can only produce symptomatic relief and have their own limitations and long-term side effects. Therefore, the need for alternative therapy is the need of the hour. Fetal nigral striatal grafts and neural stem cells are successful candidates used in the last few decades as a choice for PD. However, due to the fetal source it has ethical, immunological, tumorigenic risks besides sourcing concerns [8]. This has led scientists to explore further into the capacity of adult stem cells as a therapeutic target for PD since these are proven to be relatively safe, free of ethical issues, do not form tumours and have immunomodulatory potential. Studies have proved that both embryonic and adult stem cells *in vitro* can be transdifferentiated into functional dopamine secreting cells [14, 21, 22]. Animal data suggests that it is possible to transplant these cells into the brain and have therapeutic benefits in PD [23–25]. Hence, in this study we have chosen adult bone-marrow-derived mesenchymal stem cells.

In our first study [19], we have demonstrated the safety of autologous bone-marrow-derived mesenchymal stem cells transplanted unilaterally into the SVZ. However, with mixed results. Some patients showed improvement. There was an initial improvement period followed by a deterioration of the symptoms. This was possibly due to the continued degeneration in the nongrafted side. To nullify this effect, in the current study, we have undertaken bilateral stem cell transplantation.

During the earlier study we also noted that there is a difference in the population doubling time (PDT), morphology, differential potential, and cell senescence. Although the

Table 3: MR tractography: (a) Average ± SD of Levels of fractional anisotropic values (FA) in 12 different sites in brain of PD patients. (b) Average ± SD of Levels of fractional anisotropic values (FA) in 12 different sites in brain of PD plus patients.

(a)

Site	FA values			
	Baseline	1st follow up	2nd follow up	3rd follow up
CSO-right	0.48 ± 0.09	0.46 ± 0.07	0.48 ± 0.15	0.50 ± 0.02
CSO-left	0.48 ± 0.07	0.48 ± 0.10	0.47 ± 0.11	0.51 ± 0.13
AL-right	0.42 ± 0.08	0.42 ± 0.09	0.36 ± 0.05	0.48 ± 0.19
AL-left	0.40 ± 0.07	0.36 ± 0.06	0.38 ± 0.08	0.33 ± 0.04
PL-right	0.66 ± 0.06	0.57 ± 0.14	0.64 ± 0.09	0.61 ± 0.16
PL-left	0.65 ± 0.11	0.60 ± 0.12	0.65 ± 0.07	0.61 ± 0.24
Genu-right	0.53 ± 0.13	0.56 ± 0.11	0.50 ± 0.16	0.69 ± 0.07
Genu-left	0.54 ± 0.14	0.58 ± 0.07	0.51 ± 0.11	0.63 ± 0.14
SPL-right	0.67 ± 0.12	0.69 ± 0.06	0.66 ± 0.11	0.65 ± 0.08
SPL-left	0.65 ± 0.19	0.73 ± 0.03	0.69 ± 0.10	0.59 ± 0.27
Peduncles-right	0.52 ± 0.13	0.53 ± 0.07	0.61 ± 0.08	0.56 ± 0.08
Peduncles-left	0.50 ± 0.14	0.56 ± 0.08	0.55 ± 0.05	0.64 ± 0.04

(b)

Site	Baseline	Follow up
CSO-right	0.3995 ± 0.0881	0.395 ± 0.07125
CSO-left	0.43125 ± 0.398	0.44575 ± 0.08
AL-right	0.398 ± 0.0735	0.38475 ± 0.160
AL-left	0.3875 ± 0.0723	0.3515 ± 0.1135
PL-right	0.649 ± 0.0893	0.59475 ± 0.099
PL-left	0.66775 ± 0.079375	0.604 ± 0.10085
Genu-right	0.378 ± 0.1255	0.3555 ± 0.1219
Genu-left	0.433 ± 0.100	0.425 ± 0.1336
SPL-right	0.61 ± 0.1413	0.629 ± 0.118
SPL-left	0.63675 ± 0.14315	0.67 ± 0.13015
Peduncles-right	0.555 ± 0.126	0.60725 ± 0.149
Peduncles-left	0.512 ± 0.1016	0.53 ± 0.12065

cells met the required standards of the ISCT, the PDT, and cell surface marker expression and differential potential was observed to be lesser than healthy donor BMMSCs. This may be attributed to the higher age of the patient where cells are known to have shorter telomere length [23] and lower proliferation potential. These cells also reached senescence *in vitro* much earlier (Passage 3) and hence it was challenging to be able to upscale the cells for transplantation (unpublished data). The mixed results obtained may be attributed to the differences in the cell properties observed.

In view of the preceding results, in this study we wanted to understand the safety and feasibility of bilateral "allogenic" bone-marrow-derived mesenchymal stem cells for PD. The rationale was to rule out bone marrow aspiration in the aging population of PD patients and the morbidity associated with it. In the current study, we have transplanted allogenic healthy donor mesenchymal cells at passage 2. These cells are easy to upscale *in vitro*, maintain differential potential and cell surface marker expression. It is believed that these cells will have potentially higher therapeutic benefits. Since these cells can be produced in a large scale, cell expansion would also help to make the therapy more affordable. And bilateral transplantation would prevent any further degeneration on the contralateral side.

After receiving appropriate approvals, the study was conducted in 8 PD patients and 4 PD plus patients. The small number was chosen to understand the safety of injecting allogenic adult bone marrow mesenchymal stem cells into the subventricular zone of PD patients.

4.1. Our Hypothesis. Parkinson's disease involves both the nigral and extranigral systems. As a result, there are motor complications, associated dementias, multiple system dysfunctioning, and decline in cognitive functions with time.

Most studies have focused on the motor aspects only which are due to the loss of dopaminergic neurons (DA) in the substantia nigra of the midbrain. Cell replacement experiments conducted till date are targeted towards the replacement of the DA neurons. The results of fetal mesencephalic transplantation show graft induced dyskinesias due to

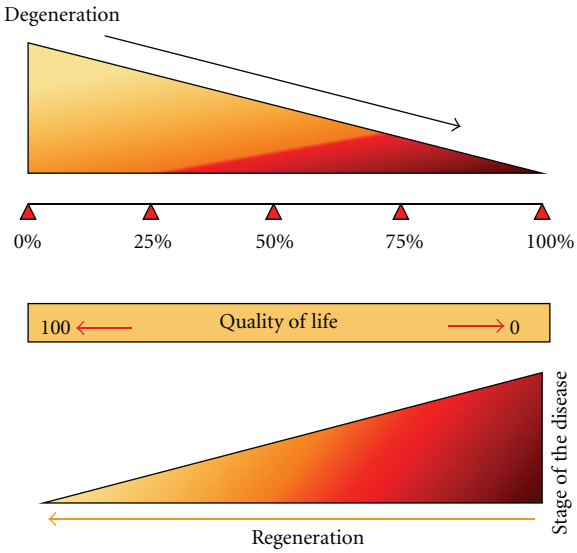


Figure 5: Depicts the balance between degeneration due to illness and the neuroregenerative reserve present in the brain. The schematic diagram illustrates that higher the degeneration higher the illness and more the regeneration required. Exogenous supply of stem cells midway between these two stages (i.e., 50%) would aid in improving the quality of life and reducing the disability due to disease.

inflammation around the implants, mixed population of DA neurons, and inappropriate synaptic contacts [6, 8–10].

Therefore, in our study we opted to choose a cell type which would primarily help in neuroprotection of the affected region irrespective of the cell type, followed by neurogenesis. This is a more global approach to the problem which would target not only the classical motor symptoms but also associated memory loss and decline of cognitive functions. Our primary aim was to forestall the progress of the disease and secondly help in restoration of neural functions. This is the first paper to demonstrate that bilateral allogenic transplantation of adult bone-marrow-derived mesenchymal stem cells is safe and has beneficial neuroprotective and neurorestorative effects in PD patients.

In this context, it is imperative to understand how mesenchymal stem cells help in neuroprotection and neurogenesis.

It is well established that BMMSCs are capable of releasing cytotrophic mediators such as nerve growth factor (NGF) superfamily-Brain-derived neurotrophic factor (BDNF) and NGF-3; Glial-derived neurotrophic factor (GDNF) and neurturin [26]. These neurotrophic factors are essential for neurogenesis, neuroprotection, neuronal survival and differentiation. In PD animal models-mesenchymal stem cells are known to slow the progress of degeneration, improve neighbouring neuronal activity, regenerate nerve fibres, and most significantly induce proliferation and differentiation of the resident pool of neural stem cells [27].

Data from animal studies also demonstrate that inflammation at the SN of the midbrain leads to significant loss of dopaminergic neurons. Also there is a noted increase in the levels of tumour necrosis factors-α, interleukin 1β and γ interferon in the SN of PD patients [28]. Mesenchymal stem cells are known immunomodulatory. *In vitro* studies show that they are involved in immunosuppressive activities although the mechanism of action needs further clarity. In autoimmune encephalomyelitis animal models, mesenchymal stem cells have demonstrated a reduction in inflammatory infiltrates, lesser relapses, and neural insults. Recent studies are suggesting that NSAIDs are said to have beneficial effects in PD patients. An anti-inflammatory beneficial effect of mesenchymal stem cells is being proposed here.

The neural stem cells are located in the subventricular zone of the brain and hence is the most preferred site of injection in this study although invasive. The exogenous BMMSCs would help to activate and increase proliferation of the resident stem cells which has regenerative capacities (endogenous regeneration). At the same time, SVZ is far away from the known lesioning targets in the brain.

In this study, we report that there is 22% improvement in the UPDRS scores of the patients treated. The improvement was noticed only in the early diseased patients and not in PD plus. At the end of the study (12 months) it was not required to increase the medication which is an indicator that disease progression has been prevented. Further follow-up studies are on-going. No study till date has reported the ability to stall progression of PD in patients. Concurrent to the UPDRS scores, there was a sense of subjective well being perceived by the patient and caregivers in 10 out of 12 patients.

For PD plus, 3 patients have shown slight transient improvement post stem cell transplantation. In 1 patient there was no noticeable change. We feel that BM-mesenchymal stem cells should be considered as a treatment of choice in early-stage PD patients to appreciate maximum benefits. This is due to the loss of the "neuroregenerative reserve" present in the brain. It proves the fact that once the disease has progressed further and involves multiple areas, it is difficult to stop the process. Also the degeneration is so extensive and rapid that it is beyond the reparative capacity of the exogenous bone marrow mesenchymal stem cells to help in neuroprotection and neurogenesis. We also assume that the degeneration process is slow in the initial phases and gains momentum with time. PD plus appears to be involving multiple areas of degeneration *de novo* and hence none of the drugs or surgery are useful. Unfortunately cell therapy does not seem to alter the course. This creates a need for further studies where we need to consider the option of providing multiple doses of cells at frequent intervals and/or test the potential of stem cells derived from a different source like adipose tissue or umbilical cord matrix as these are also known to possess neuroprotective effects and higher transdifferentiation potentials. At baseline and 12 months after stem cell transplantation, MRI of the brain was performed. Although there has been improvement in symptoms and no further progression of the disease, there were no structural changes observed in the MRI scan. The MR tractography results show a specific pattern of recovery. Certain structural changes were observed in the genu of the corpus callosum and the left peduncle suggesting that early regeneration of the tracts probably occurs here. These changes are persistent throughout the follow-up study and clinically correlated with

improvements reported in the patients. Currently we are continuing the study, with PET scans which will give us valuable information on any metabolic and functional changes happening in the SN region of the midbrain. This would give us further valuable clues in to the mechanism of regeneration in the human PD-affected brain.

Thus, to summarize, BM-mesenchymal stem cells have a three pronged therapeutic approaches in neurodegenerative diseases such as PD: neurogenesis, neuroprotection and neural plasticity. Also, it is essential to expose the degenerating brain to the exogenous stimulus of stem cells, while the *in situ* neuroregenerative reservoir of stem cells is present that is, in the early part of the disease.

5. Conclusion

This is the first paper to demonstrate that bilateral allogenic transplantation of adult bone-marrow-derived mesenchymal stem cells is safe and has beneficial neuroprotective and neurorestorative effects.

The study establishes the safety of adult bone-marrow-derived mesenchymal stem cell transplantation bilaterally into the subventricular zone of the human brain using burr hole surgery. There are improvements in the UPDRS scores of the PD patients, reported subjective well being and no increase in medications during the follow-up period. It is to be noted that no improvements were observed in the PD plus patients. This strengthens the fact that stem cell transplantation in the early stages of PD has the potential to prevent further progress of the disease. Results from this study suggest that allogenic BM-mesenchymal stem cells may be used as a disease modifying therapeutic strategy in treating PD. Unlike the known indications for surgical intervention, we recommend intervention in the early part of the disease to reap the best benefits. However, further long-term follow-up studies need to be carried out to understand the long term safety and sustainability of the benefit. Currently studies are going on to elucidate the mechanism of action of these cells in neuroprotection and neurogenesis in PD-affected human brain.

Conflict of Interests

The authors report no conflicts of interest and are responsible for the content and writing of the paper.

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Review Article

Mesenchymal Stem Cells as a Potent Cell Source for Bone Regeneration

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While small bone defects heal spontaneously, large bone defects need surgical intervention for bone transplantation. Autologous bone grafts are the best and safest strategy for bone repair. An alternative method is to use allogenic bone graft. Both methods have limitations, particularly when bone defects are of a critical size. In these cases, bone constructs created by tissue engineering technologies are of utmost importance. Cells are one main component in the manufacture of bone construct. A few cell types, including embryonic stem cells (ESCs), adult osteoblast, and adult stem cells, can be used for this purpose. Mesenchymal stem cells (MSCs), as adult stem cells, possess characteristics that make them good candidate for bone repair. This paper discusses different aspects of MSCs that render them an appropriate cell type for clinical use to promote bone regeneration.

1. Introduction

Bone is a highly specific, dynamic tissue capable of maintaining viability under mechanical stress and external continuous compression. This capability of bone tissue diminishes with increasing age [1]. Furthermore, small bone damage can repair spontaneously without intervention. However, if there is extensive bone damage due to pathologic and traumatic injuries, there will be a need for reconstructive surgery and bone transplantation. In this regard, autologous tissue transplantation would be the best and safest strategy for bone repair. Autologous bone graft is taken from the patient's own iliac crest, ribs, or calvarium. Unfortunately, access to autologous bone graft is limited. Furthermore, obtaining an autograft is associated with morbidity, pain, and infection at the donor site. Because of such disadvantages other alternatives are needed [2, 3]. Allogenic bone tissue implantation may be chosen to repair large bone defects, but this bone substitute also exhibits several drawbacks, which include the possibility of disease transmission, graft rejection, problems with graft integration and viability at the recipient site [4]. Emergence of modern bone engineering strategies based on osteogenic cells, osteoinductive stimulator, and osteoconductive scaffolds are recognized as potential ways to create biologic tissue substitutes for regenerating large bone defects [5]. The choice of cell sources that can efficiently differentiate into bone tissue is the first, important step during bone engineering. Several cell types can potentially be used as cellular components in bone engineering. These include osteoblast, embryonic, and adult stem cells. Among these candidates, mesenchymal stem cells (MSCs) as adult stem cells possess some characteristics that make them more appropriate for use in promoting bone regeneration.

Historically, the definitive presence of MSCs was discovered about 40 years ago by Friedenstein et al. in bone marrow tissue. They described these cells as mononuclear nonphagocytic cells with fibroblast-like phenotype and colongenic potential capable of adhering to the culture surface in a monolayer culture [6]. Later, it has been shown that MSC-like population were present in a wide range of adult tissues, including trabecular bone [7], synovium [8], adipose tissue [9], skeletal muscle [10], periosteum [11], dermis [12], blood [13, 14], deciduous teeth [15], amniotic fluid [16], and umbilical cord blood [17]. Currently, good manufacturing

practice (GMP) has been developed to produce the cells for use in clinic [18].

It should be mentioned that stem cells are defined by two key characteristics: the ability of multilineage differentiation and the capacity of self-renewal [19]. Of these MSCs possess multilineage differentiation potential but have a limited proliferation capacity since they enter senescence after a few population doubling in culture [20, 21]. Therefore they cannot be considered true stem cells. For this reason, in related literatures the cells have been referred to as by different terminology as colony-forming unit fibroblasts (CFU-Fs), mesenchymal stromal cells (MSCs), marrow stromal cells (MSCs), marrow progenitor cells (MPCs), and marrow stromal fibroblasts (MSFs) [22–28]. Nowadays, the term mesenchymal stem cells is the dominant term most frequently used by investigators. Here, the specific characteristics that make MSCs promising cells for use in bone regeneration strategies will be discussed.

2. MSCs Escape Ethical Concerns

Among candidate cells for bone regeneration, embryonic stem cells (ESCs) possess ethical issues limiting their application in bone regeneration. ESCs are derived from the blastocyst inner cell mass and can be directed toward differentiation into varying cell lineages, including osteoblastic cell lineages under suitable culture conditions [29–32]. To date, multiple studies have been conducted on ESCs osteogenic differentiation in vitro and their application in bone tissue engineering with varying scaffolds. For example, it has been shown that culturing ESCs on poly-lactide-co-glycolic (PLGA) or nanofibers made from PLLA (poly (l-lactic acid)) is associated with high expressions of osteogenic markers, including alkaline phosphatase and osteocalcin [33, 34]. Despite increasing interest in the application of ESCs in bone engineering technology, research is highly limited due to political issues as well as ethical concerns associated with these cells. The primary concern is the source from which these cells are derived. The use of excess embryos produced in IVF to create ESCs is not acceptable according to religious and ethical points of view. Additionally, some reports have indicated that transplantation of ESCs has led to teratoma formation in the animal model [35, 36]. For these reasons postnatal adult stem cells, including MSCs that could be derived from a patient's own tissues and do not possess ethical limitations, are considered more appropriate for clinical use.

3. MSCs Are Residents of Multiple Tissues

MSCs have been reported to constitute about 0.01%–0.001% of the marrow mononuclear population [37]. These cells can be isolated from marrow aspirates of the superior iliac crest, femur, and tibia. For this purpose, marrow cells are usually enriched for mononuclear cells with Ficoll or Percol and then plated on culture plastic vessels in order to prepare adherent cell populations [38]. It has recently been demonstrated that late plastic adherent MSCs possess higher osteogenic potential [39]. Alternatively, MSCs can be obtained by the preparation of a population positive for STRO-1 or CD105. It has been reported that a population negative for CD45 or Gly-A are from MSCs [40]. By now, many researchers have studied optimized culture and differentiation of MSCs in vitro and their application in regenerating bone defects in animal models and humans [41–44]. Since collection of bone marrow is invasive and expansion and osteogenic differentiation of marrow-derived MSC seem to be reduced with advancing age, investigators have attempted to find other tissue sources for MSCs [45]. According to research, multiple tissues have been found to contain MSC-like population; of these, adipose tissue as well as birth-associated tissues, including umbilical cord and dental pulp, has gained considerable attention.

The presence of cells with multipotent differentiation capacity in adipose tissue is promising due to the ease of accessibility of adipose tissue and its abundance in the body. Adipose tissue can be an appropriate substitute for marrow in regenerative medicine and tissue engineering [46, 47]. Adipose-derived stromal cells (ADSCs) can be derived from adipose collected by liposuction and lipectomy [48]. ADSCs are able to maintain proliferation potential as well as differentiation capacity even in older people. The differentiation potential of ADSCs is largely dependent on the concentration of ascorbic acid and dexamethasone in culture medium [49, 50]. By now, many studies conducted on animal models have confirmed the regenerative potential of ADSCs in bone defects. The first report regarding repair and production of bone tissue in vivo belongs to Lee et al. who transplanted ADSCs loaded onto PLGA [51]. Later, Hicok et al. have noted the production of osteoid matrix when a combination of ADSCs, hydroxyapatite (HA) and tricalcium phosphate (TCP), were transplanted in nude mice [52]. In 2004, these cells were used for the repair of human calvarial defects [53]. To date the effect of various biomaterials, including HA, human cancellous bone fragments, deproteinized bovine bone granules, and titanium, has been investigated in terms of ADSC attachment, proliferation, and differentiation [54, 55].

The umbilical cord from a newborn baby contains two arteries and a vein covered with mucus connective tissue rich in hyaluronic acid, referred to as Wharton's jelly. According to studies, MSC-like cells can be derived from various components of this cord [56]. For example, blood from an umbilical cord is a rich source for pluripotent cells which are also referred to as umbilical-cord-blood-derived MSCs (UCB-MSCs). These cells are quite similar to marrow-derived MSCs and have osteogenic potential in an optimized culture [57–59]. Many investigations have thus far been conducted on bone engineering by using these cells and various scaffolds [60, 61].

Several stem cell types in dental tissue have been reported including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells of the apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle progenitor cells (DFPCs) [15, 62]. Since DPSCs can be easily isolated by enzymatic digestion of pulp tissue many studies have been conducted

regarding bone engineering with these cells and appropriate 3D scaffolds, including HA/TCP and polylactic-co-glycolic acid (PLGA) [63, 64].

4. MSCs Can Efficiently Differentiate along an Osteogenic Lineage

MSCs osteogenic property was the first reported differentiation capacity when they were discovered. Indeed, even prior to definitive isolation of MSCs from bone marrow, some transplantation experiments clearly showed the osteogenic capacity of marrow tissue. Friedenstein et al. were the first to isolate and describe the cellular equivalent of osteogenic features of marrow tissue [65].

Osteogenic differentiation is a highly programmed process that consists of many stages including proliferation, differentiation, matrix deposition, mineralization, and matrix maturation. The general protocol for in vitro bone differentiation of MSCs involves incubation of cell monolayer in a culture medium containing dexamethasone, beta glycerol phosphate, and ascorbic acid for a period of two to three weeks [66]. Dexamethasone is a synthetic glucocorticoid that stimulates MSC proliferation and is essential for their osteogenic differentiation [67, 68]. Although the mechanism of dexamethasone's effect is not well known, it has been speculated that this reagent exerts its effects through upregulation of the beta catenin-like molecule TAZ, which results in up-regulation of Runx2-related transcription factor and osteogenic differentiation [69]. The optimal concentration of this reagent for MSC bone differentiation is about 10 nM, which corresponds with its physiologic concentrations [70]. Organic phosphate released after enzymatic hydrolysis of beta glycerol phosphate plays an important role in matrix mineralization. This free phosphate is usually applied in 5–10 mM concentrations for MSC bone differentiation [71]. Ascorbic acid is a cofactor in the hydroxylation of prolins and lysine moiety of collagen molecules and is the abundant protein in ECM. This reagent is used in 50–500 μM concentrations [72]. In addition to these osteogenic supplements, there are other osteogenic factors including (1,25-D3) 1,25-dihydroxyvitamin and BMPs (BMP2) [73].

MSC in vitro bone differentiation results from the activation of some well-known molecular signaling pathways. Each osteogenic reagent activates a molecular pathway that leads to a differentiated phenotype. Although the osteogenic effects of a number of these reagents have long been known, specific pathways by which the effects are mediated remain to be clarified. The activation of wingless-type MMTV integration site family of the protein (Wnt) signaling pathway [74], mitogen-activated protein kinase (MAPK) signaling pathway [75], TGF beta and BMP signaling pathways [76], and RHO-GTPase signaling pathway [77] has been established in MSC bone differentiation.

Activation of signaling pathways by osteogenic supplements eventually leads to activation of osteoblast-specific signal proteins and specific osteoblastic transcription factors. Cbfa1 (core binding factor alpha 1) also called as Runx2 (runt-related gene 2) is one of the most studied transcription factors expressed in MSCs upon their commitment toward osteogenic differentiation [78, 79]. Runx2, as a master switch, adheres to osteoblast-specific cis-acting element (OSE2) in the gene promoter region stimulating the expression of bone-specific genes such as coll I, osteocalcin, osteopontin, and alkaline phosphatase [80]. Osterix is another transcription factor involved in MSC bone differentiation, which has been clearly shown in murine MSCs where they were retrovirally transduced with the osterix gene [81]. Addition of dexamethasone to the culture of murine calvarial osteoblasts has been reported to induce expression of osterix as well as Runx2 genes [82].

Optimal conditions for MSC in vitro bone differentiation are well been established. For example, the addition of rhBMP-2 to osteogenic medium can facilitate proliferation and osteogenic differentiation of BMSCs both in vitro and in vivo. The use of alpha MEM versus DMEM and application of low-passaged versus high-passaged cells can end with higher expression of osteogenic genes and more culture mineralization [83–85]. Studies on scaffold designing and the effect of biomaterial on bone repair have indicated that calcium phosphate-based scaffolds, including hydroxyapatite (HA) and tricalcium phosphate (TCP), are more appropriate for bone engineering due to their osteoconductive properties [86]. Applying fluid shear stress (FSS) on the MSC osteogenic culture increases the expression of bone-specific genes and deposition of mineralized matrix. FSS mediates its effects through regulation of mechanosensitive signaling molecules, including ion channel and integrins, which are able to convert mechanical into chemical signals [87, 88].

5. Nonimmunogenic Properties of MSCs

MSCs possess immunologically specific characteristics; therefore they would be general donors for therapeutic applications. Immunologic phenotypes of MSCs are MHC I+, MHC II−, CD 40−, CD80−, and CD 86− [89]. Graft rejection by the immune system occurs when T cells are fully activated. T cells require two signals to become fully activated. The first signal is provided through the T-cell receptor which interacts with peptide-MHC molecule 1 on the membrane of antigen-presenting cells (APC). A second signal, the costimulatory signal, is provided by the interaction between co-stimulatory molecules, including CD80 and CD86 that are expressed on the membrane of APC and the T cell [90]. MSCs do not trigger T-cell activation owing to the absence of CD80 and CD86 in their membrane [91]. The immunosuppressive nature of MSCs has been shown in skin allografts of baboon models.

According to research, MSCs secrete soluble factors that inhibit CD4+ and CD8+ T-cell activation as well as proliferation. Among these factors are indoleamine 2,3-dioxygenase (IDO), nitric oxide, TGF-beta, and prostaglandin E 2 [92–95]. It has been demonstrated that MSCs stop the B-cell cycle at the G0/G1 stage and inhibit their differentiation into plasma cells [96, 97]. Ramasamy et al. have indicated that BMSCs are able to inhibit dendritic cell (DC) differentiation and prevent them from entering into the cell cycle [98].

DCs are able to efficiently present antigens to lymphocytes. According to research, monocytes differentiate into DCs in the presence of MSCs [99].

Immunomodulatory features of MSCs make them an important cellular candidate for cell-based treatment of tissue defects in an allogenic setting. For this reason, there is hope that MSCs could replace autologous and allogenic bone grafts which have known exhibited limitations in terms of availability and risk of pathogen transmission, respectively. At the allogenic approach, it will be possible to develop a cell bank to maintain MSCs from every donor for use in cell therapy. Prior to routine application of the cells in the clinic, an exact understanding of the immunologic features of MSCs and the underlying mechanism of action is needed [100, 101].

6. Injury-Seeking Capability of MSCs

One of the most important capabilities of MSCs is their migration capacity in response to signals produced by an injured bone [102, 103]. At the injury site, MSCs could possibly help with repair in two ways: (1) they differentiate to tissue cells in order to restore lost morphology as well as function, and (2) MSCs secrete a wide spectrum of bioactive factors that help to create a repair environment by possessing antiapoptotic effects, immunoregulatory function, and the stimulation of endothelial progenitor cell proliferation [103].

The precise mechanisms of cell trafficking in blood, transmigration through endothelial cell, and homing to the injured site are not thoroughly understood, but it has been speculated that chemokines and their receptors regulate this process [89]. Chemokines (chemotactic cytokines) are small proteins (8–10 KDs) with a capacity for creating a chemical environment appropriate for the migration of lymphocytes, neutrophils, and other immune cells towards inflammation, angiogenesis, and the organogenesis site. On the other hand, MSCs express a series of chemokine receptors that play a role in their migration in response to a chemokine gradient produced at the damaged site. These chemokine receptors include CCR1, CCR7, CCR9, CCR3, CCR4, CCR5, and CX3CR1 [104]. CXCR4 receptor and its specific chemokine (stromal cell-derived factor 1 (SDF1)) play an important role in stem cell trafficking, particularly HSCs [105]. It has been proposed that SDF1/CXCR4 could be a homing signal for MSCs in bone repair.

Kitaori et al. have reported that SDF1 expressed by periosteum mediates bone repair in the murine femoral model by recruiting MSCs to the fracture site [106]. The SDF1 gradient causes both host as well as infused MSCs to migrate towards the injured area. MSC migration has been proven in clinical trials performed by Horwitz et al. in which MSCs were injected to regenerate bone in six patients who suffered from osteogenesis imperfecta, where osteoblasts secrete defective collagen I resulting in osteopenia. Observations indicated that in 5 out of 6 children who received allogenic MSCs, cell migration to various tissues that included bone, skin, and marrow stroma was observed [107]. Transplantation of MSCs was followed by increased formation of compact bone and reduction in fracture frequency.

Considering the relationships of cell migration with the chemokine concentration gradient, it can be concluded that the application of MSCs must be performed at the time when the chemokine concentration gradient is established at an adjacent area to the injured site.

7. MSCs as Vehicles for Bone Gene Therapy

MSCs could be ideal carriers for therapeutic genes at a cell-mediated gene delivery strategy owing to their unique characteristics that include ease of isolation, culture, and expansion as well as their immunomodulatory property [108].

In the normal process of bone development and repair, cytokines and osteoinductive growth factors play a major role by recruiting osteogenic progenitors at the bone formation site and promoting their differentiation into bone cell lineages [109]. Therefore, the application of such factors which include related recombinant growth factors in large areas of bone damage would enhance new bone formation. However the problem is that recombinant growth factors have a limited half-life that limits their sustained supply into damaged tissue. To overcome this limitation, gene transfer strategies using cellular carriers have been proposed. This strategy offers the sustained delivery of the osteogenic factor to the damaged area [110]. Genetic manipulation of MSCs can be achieved by transduction using viral vectors such as the adenovirus (Ad) [111] or transfection by nonviral vectors such as liposomes [112]. Viral vectors have the advantage of high efficiency but trigger the immune system. In addition, they possess varying capacity to transfer genes into dividing and nondividing cells [113]. Non-viral vectors possess the advantage of not being toxic [114].

Many investigators have tried to regenerate bone by transfecting MSCs with the BMP gene. For example, Lieberman et al. have indicated that autologous BMSCs expressing Ad-BMP2 can considerably promote segmental femoral defects in rat models when compared with BMSCs expressing Ad-LacZ [115]. Transplantation of Ad-BMP2-MSCs in rabbits has been reported to be associated with new bone formation [116]. In spite of the multiple studies that have focused on temporary expression of factors using the adenovirus vector, Gysin et al. have observed permanent expression of BMP4 using retrovirus in BMSCs which lead to repair of critical sized calvarial defects in rats [117]. In one study, Lin et al. have compared BMP4-transfected MSCs from marrow and adipose tissue in bone repair of a rabbit model and found no significant difference [118].

It has been shown that Ad-Runx2-MSCs transplanted in murine calvarial defects produce more bone tissue compared to MSCs [119]. Recent studies have focused on simultaneous application of BMPs and RUNX2. When these two factors were entered into an immortal MSCs line and injected into mice, considerable bony ossicle with marrow cavity was observed (compared to the application of cells that expressed Ad-BMP2) [120]. Although no clinical trial to date has been conducted using genetically modified MSCs, studies have

indicated that such a strategy would be more effective in enhancing bone repair.

8. Conclusion

MSCs as adult stem cells are free from ethical concerns, residents of multiple tissues, able to efficiently differentiate along an osteogenic lineage, possess non-immunogenic properties, have injury-seeking capabilities, and can be used as vehicles for bone gene therapy. These characteristics make MSCs safe and promising candidates for use in bone engineering and regeneration. Currently, several clinical trials are being performed on problematic human bone lesions, including nonunion fractures, delayed union, bone cysts, and bone neoplasms, among others. These ongoing registered trials are available at the following clinical trial website: http://clinicaltrials.gov/.

Conflict of Interests

None of the authors have conflict of interests to declare.

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Review Article

Mesenchymal Stem Cells and Cardiovascular Disease: A Bench to Bedside Roadmap

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In recent years, the incredible boost in stem cell research has kindled the expectations of both patients and physicians. Mesenchymal progenitors, owing to their availability, ease of manipulation, and therapeutic potential, have become one of the most attractive options for the treatment of a wide range of diseases, from cartilage defects to cardiac disorders. Moreover, their immunomodulatory capacity has opened up their allogenic use, consequently broadening the possibilities for their application. In this review, we will focus on their use in the therapy of myocardial infarction, looking at their characteristics, *in vitro* and *in vivo* mechanisms of action, as well as clinical trials.

1. Introduction

Although traditionally regarded as a health concern related particularly to the industrialized world, cardiovascular diseases are now the first cause of death worldwide [1], with myocardial infarction (MI) resulting in 12.8% of deaths. Aside from changes in ways of life associated with economic and social development, one of the main reasons is the fact that MI is an evolving disease. After the ischemic event, anaerobic conditions rapidly induce massive cell death, not only involving cardiomyocytes (CMs), but also vascular cells. Although the organism tries to exert a compensatory activity (reviewed in [2]) during the first stages of the disease and may even manage to partially restore functionality, the resulting scar is never repopulated, relentlessly leading the patient towards the setting of heart failure. Thus, though not conventionally regarded as such, cardiac disease is a degenerative affection in which lack of sufficient contractile and vascular cells leads to a decompensated neurohormonal microenvironment [3], which further impairs both organ function and cell survival.

Although the existence of stem cells has been a well-known fact for nearly half a century [4], it is in the last 15 years that the field has experienced a major boost. Their capacity for differentiation has made stem cells outstanding candidates for the treatment of degenerative diseases, substituting for cells lost during the course of the disorder. Consequently, cardiac diseases and MI have been the object of intense research [5]. Among the cell types studied, mesenchymal stem cells (MSCs) are strong candidates for success in the MI setting. In the following pages, we will discuss their capacities as well as pre- and clinical investigations in which these cells have been employed.

2. Origin, Types, and Characteristics

The studies by Friedenstein and colleagues are regarded as one of the first reports on MSC [4]. In these, the clonogenic potential of a population of bone marrow- (BM-) derived stromal cells, described as colony-forming unit fibroblasts, was examined. BM is indeed one of the best-known sources of progenitor cells, MSC being among them [6]. Although this is not entirely understood, BM-MSC are thought to act as supporters and nurturers of other cells within the marrow [7–9], possibly in a location close to blood vessels [10]. However, there is a relatively small population (0.01%–0.0001% of nucleated cells in human BM [11]), so MSC can be easily purified by plastic adherence and expanded after BM extraction. Similarly, but adding

simple mechanical and enzymatic processing, a mixed cell population (called stromal vascular fraction, SVF) can be isolated from adipose depots, which, after *in vitro* culture and homogenization, gives rise to the mesenchymal progenitors from this tissue, also termed adipose-derived stem cells (ADSCs) [12]. Adipose tissue is regarded as a much richer source of progenitors, harboring 100 to 500 times the numbers seen in BM [13]. However, despite similarities in phenotype, differentiation, or growth kinetics, there are certain differences at a functional, genomic, and proteomic level [9, 14], suggesting a degree of higher commitment of BM-MSC to chondrogenic and osteogenic lineages than ADSC [15].

Adipose tissue and BM are the most widely researched sources of mesenchymal progenitors because they are easy to harvest, and owing to the relative abundance of progenitors and the lack of ethical concerns. Nevertheless, MSCs have been ubiquitously found in a variety of locations, as umbilical cord blood [16], dental pulp [17], menstrual blood [18], or heart [19], among others (reviewed in [20]). This wide variety of origins, methodologies, and acronyms prompted standardization in 2005 by the International Society for Cellular Therapy, which set the minimum requirements for MSC definition (Table 1). First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [21]. Still, caution must be taken as some reports fail to meet these criteria, and MSC is often employed for "marrow stromal cell," "mesenchymal stromal cell" or "marrow stem cell." Accordingly, a clarification was published in which MSC was defined as "multipotent mesenchymal stromal Cells" [22], adding the supportive property to the required characteristics [23].

Table 1: Standardized requirements for MSC definition.

Multipotent mesenchymal stromal cells (MSE) properties
(i) Plastic adherence
(ii) Cell surface antigen expression profile
$CD73^+$, $CD90^+$, $CD105^+$, HLA-DR^-, $CD11b^-$, $CD14^-$, $CD19^-$, $CD34^-$, $CD45^-$, $CD79\alpha^-$
(iii) Multipotency
Chondroblast, Adipocyte, Osteoblast

3. What Do MSCs Have to Offer to Cardiac Regeneration?

When considering the goal of cardiac tissue regeneration, the desired objective must encompass three objectives: (i) the production of a replacement myocardial mass, (ii) the formation of a functional vascular network to sustain it, and (iii) the returning of the impaired ventricle to its proper geometry. Cell therapy may theoretically affect those processes in two ways: either by direct differentiation of transplanted cells towards the desired lineages or by their production of molecules with therapeutic potential (Figure 1).

BM-MSC have shown their *in vitro* capacity to give rise to endothelial cells (ECs) [24, 25] and smooth muscle cells (SMCs) [24]. Cardiomyocyte differentiation has proved more problematic, as either demethylating agents have been employed [26], or it has been inefficient and incomplete [27, 28]. In contrast, the cardiac potential of ADSC is better documented *in vitro*, showing their capacity to give rise to CM, either by the use of DMSO [29] or CM extracts [30]. In addition, ADSC seems to harbor a progenitor subset characterized by the expression of Nkx2.5 and Mcl2v [31] and whose differentiation relies on the autocrine/paracrine activity of vascular endothelial growth factor (VEGF) [32]. SMC [33] and EC [34] have been obtained from adipose cells, yet a cautionary note must be struck, as some of these studies either rely on subpopulations of freshly isolated cells or culture them in differentiation-promoting medium before purifying the mesenchymal population [35, 36]. Finally, other mesenchymal progenitors have also been differentiated to CM or CM-like cells, such as menstrual blood-derived MSC [18] or umbilical cord blood MSC [37].

However, although it is extremely interesting, this differentiation potential must cope with two opposing factors. First, patients receiving stem cell therapy are severely diseased and usually elderly, two factors that have an outstanding impact on stem cell function. For instance, a decrease in the numbers and functionality of circulating endothelial progenitors is directly related to cardiovascular risks and smoking [38, 39] and age has also been shown to impair the angiogenic capacity of both ADSC [40] and BM-MSC [41]. Second, the small percentage of engrafted cells (see [42] for a review) coupled to the huge catastrophe caused by an MI (the loss in some cases of over 1 billion CM [43]) and the low rate of differentiation achieved even under *in vitro* controlled conditions makes the adding of such small number of cells a therapeutically inefficient approach.

Nevertheless, secretion of beneficial molecules has been demonstrated to be able to exert a positive effect, even when a few engrafted cells are left [44]. These molecules can induce a benefit either by increasing tissue perfusion, decreasing collagen deposition and fibrosis, enhancing host-cell survival, or attracting/regulating endogenous progenitors. Thus, Chen and coworkers compared the expression profile of BM-MSC and dermal fibroblasts [45], showing that mesenchymal progenitors secreted a higher amount of several molecules, including the potent proangiogenic cytokine VEGF or the chemotactic stromal derived factor-1 (SDF-1). Conditioned medium from BM-MSC induced the recruitment of EC and macrophages, and improved wound healing. Moreover, it has recently been shown that serum-deprived BM-MSC acquire EC features and increase the release of VEGF or hepatocyte growth factor (HGF), another potent angiogenic molecule [46], both of which have been reported to be secreted by ADSC [32, 47, 48]. Moreover, Dr. March's group demonstrated that ADSCs have a pericytic nature and are able to form and stabilize functional vascular networks when mixed with endothelial

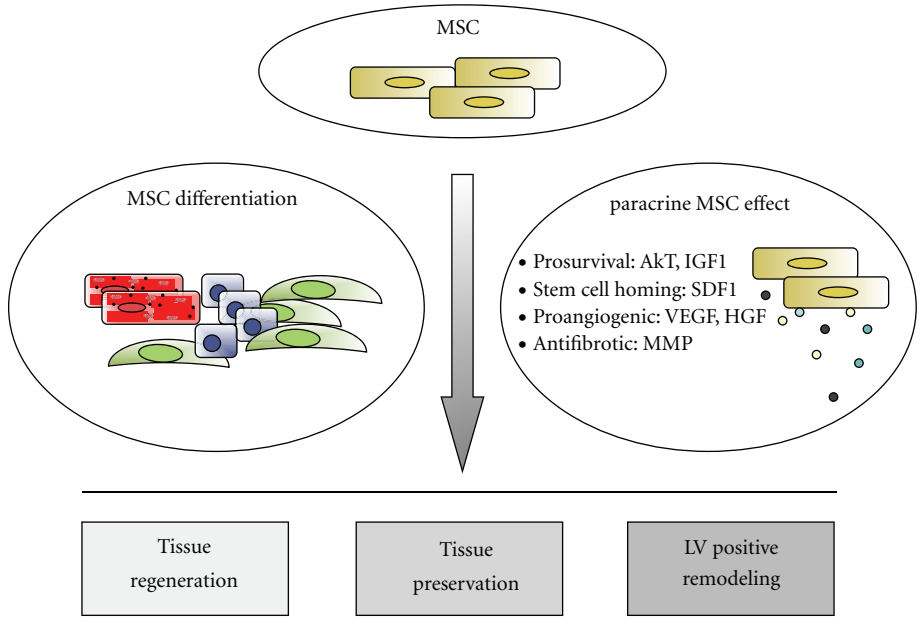

Figure 1: Main MSC actions on injured myocardium. Mesenchymal progenitors transplanted onto the ischemic myocardium are able to secrete a plethora of therapeutic molecules (paracrine activity) and even to differentiate towards (cardio-) vascular lineages, encouraging the healing of the damaged tissue, avoiding its transition to a scarred muscle, and regenerating the heart tissue mainly at the vascular level. Abbreviations: IGF-1: insulin-like growth factor-1; SDF-1: stromal derived factor-1; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; MMP: matrix metalloproteinase; LV: left ventricle.

progenitors [49]. Also, BM-MSC show a potent antifibrotic action, as their conditioned medium decreases cardiac fibroblast proliferation and expression of collagen types I and III [50, 51] and increases secretion of antifibrotic molecules such as matrix metalloproteinases (MMPs) 2, 9, and 14 [52]. These cells express five types of MMP (2, 13 and membrane type-MMP 1, 2, and 3) and are able to cross through type I collagen membranes [53], which theoretically would allow their trafficking across the infarction-derived scar. Likewise, ADSCs produce transforming growth factor- (TGF-) β1 [54], a potent regulator of fibrosis. Taken as a whole, these examples demonstrate that mesenchymal progenitors are potent paracrine mediators with a considerable capacity to impact infarct evolution.

One last noteworthy competence is the ability of BM-MSC and ADSC to modulate the immune response. Marrow-derived mesenchymal progenitors inhibit the proliferation of activated T cells and the formation of cytotoxic T cells [55], inducing an anti-inflammatory phenotype, which would allow their allogenic use and significantly broaden the scope of their applicability. However, Huang et al. reported that differentiation reduced their capacity of immunological escape [56], related to an increase of immunostimulatory molecules MHC-Ia and II and a decrease in the immuno-suppressive MHCIb. Along similar lines, McIntosh and coworkers reported that ADCS beyond passage one (and thus devoid of contaminating differentiated cells [57]) failed to elicit a response from allogenic T cells [58], but this attribute may be diminished under inflammatory stimuli, as shown *in vitro* [59].

Finally, since the onset of induced pluripotent stem cells (iPSCs) [60], mesenchymal cells have been investigated [61, 62] due to their relatively easy harvest and higher potency than other cell types (e.g., dermal fibroblasts), which show an increased efficiency, even in the absence of the oncogene c-Myc. Their supportive capacities have also made them good candidates to replace mouse cells as feeders [63, 64].

4. MSC in Animal Models of MI

However, in spite of all the positive characteristics of mesenchymal progenitors already depicted, their *in vivo* testing in animal models of the disease is compulsory. In this regard, three different settings can be found. First, the acute setting, in which cells are transplanted within hours of the MI. Here, the inflammatory microenvironment and the necrotic/apoptotic signals released from resident cells [65, 66] are the main opposing forces to the therapeutic activity of cells. Nevertheless, homing signals [67] and an antifibrotic milieu [68] may have a positive influence. Also, from a practical point of view, dealing with acute models offers the advantage of subjecting animals to only one surgery, as at the time of the MI (or minutes after it), the cells are applied, thus decreasing mortality and invasiveness. As a consequence, the majority of published reports use acute models [37, 69–84]. Most studies (with the exception of the two by van der Bogt and colleagues [74, 77]) have consistently demonstrated that the treatment induces a significant benefit for cardiac function, mainly through paracrine mechanisms that induce an increase in tissue perfusion and a decrease in the size of the scar and collagen content.

Similar results have been obtained in a second setting, the chronic one. Here, the repair processes that take place after ischemia have been completed, the scar has matured, and although a new network of blood vessels has been created, this is disorganized and inadequate [85, 86]. These facts impose a great burden upon cell survival. However, it must be taken into account that the generation of homogeneous populations as BM-MSC or ADSC needs weeks of *in vitro* culture, thus, unless used in the allogeneic setting, there is no possibility of the bedside translation of the use of mesenchymal progenitors in the acute setting. In spite of this difficulty, fewer reports deal with this issue [87–90]. Compared to results in the acute setting, mesenchymal cell therapy of chronically infarcted hearts has a positive effect upon organ contractility and histology.

As a third and intermediate position, the so-called subacute model represents a situation where angiogenic processes are still on course, either through endothelial progenitors [91] or macrophages [92], and the receding of inflammation plus the increase in fibrotic processes are also on course. As with chronic models, there are few reports in this setting [17, 18, 93, 94], but again the benefit and mechanisms appear to be consistent.

Nevertheless, analyzing in more depth the studies mentioned above, it is possible to find a fair amount of information on how mesenchymal progenitors behave when injected into the diseased heart has been gathered. Chen et al. showed that transplantation of BM-MSC into chronically infarcted rabbit hearts induced an increase in the concentration of SDF-1 that elicited the chemotaxis of host-derived BM progenitors ($CD34^+$, $CD117^+$, $STRO1^+$) and was related to a functional benefit, a decrease in infarct size and improvement in tissue vascularization [89]. Li and coworkers demonstrated that the functional enhancement was accompanied by the augmented expression of the prosurvival gene Akt [95] whereas Mias and colleagues showed that the benefit upon contractility and remodeling *in vivo* was accompanied *in vitro* by a plethora of antifibrotic actions [52]. In a sheep model of MI, the group of Dr. Spinale monitored the evolution of MMP and their inhibitors, demonstrating a relationship with the number of transplanted cells [75]. Resembling their *in vitro* behavior, several publications have demonstrated the association between proangiogenic activity *in vitro* and secretion (either direct or host-derived) of angiogenic cytokines as VEGF, HGF, or insulin-like growth factor-1 (IGF-1), among others [17, 84, 93, 96, 97]. Whether these capacities are related to the claimed pericytic nature of these cells [10, 48, 49] remains to be resolved.

Immune modulation (reviewed in [98]) in theory provides the means for the allogenic use of MSCs and as an off-the-shelf product (expanded prior to the onset of the ischemia and applicable on demand). Two reports have compared the effects of allogenic versus syngenic injection of BM-MSC in rat model of MI, with conflicting results. Imanishi et al. [78] demonstrated that both autologous and allogeneic cells improved cardiac function 4 weeks after transplantation, remained in the damaged tissues, and did not stimulate rejection. Huang and coworkers conversely [56] followed animals for up to 6 months. Syngenic cells stimulated cardiac recovery, but the effect of the allogenic treatment was transitory (significant 3 months after injection but not at 6) and BM-MSC disappeared earlier than their syngenic counterpart. However, this difference can be attributed to methodological discrepancies regarding time of transplantation (acute versus chronic resp.) or followup (1 versus 6 months). Equivalent and importantly, results from clinically relevant large animal models of MI in which allogenic cells have been employed have revealed either positive [99, 100] or no functional outcome [79]. In contrast, when autologous ADSC or BM-MSC are used [72, 83, 101, 102], reports have shown a robust and consistent functional recovery after cell transplantation. Thus, strict considerations about building up animal models must be taken into account.

5. Problems, Solutions

Despite all the optimism, stem cell therapy shows certain caveats that are amenable to improvement, namely, lack of substantial engraftment and cell persistence, high levels of death, and low *in vivo* differentiation capacity. Some approaches to try to remedy these problems have included the use of genetic manipulation and *in vitro* pretreatment of cells or biomaterials. In this sense, the CXCR4/SDF-1 axis has been greatly exploited. Ma et al. investigated the peak of cardiac SDF-1 expression [103] in rat MI, finding that injected cells at that time point (1 day postinfarction) increased cell engraftment and tissue angiogenesis. Cheng and coworkers transplanted BM-MSC engineered to overexpress the receptor CXCR4, strengthening cell homing to the injured tissue after tail vein injection [104]. The same group combined BM-MSC peripheral injection with administration of granulocyte colony-stimulating factor, which *in vitro* increased CXCR4 expression. However, although engraftment was increased, no effect of cardiac function was found [105]. Huang and associates demonstrated that overexpression of the chemokine receptor CCR1 but not CXCR2 was associated with improved survival and grafting in a mouse model of MI, which also restored functionality [106].

Cell survival in the infarcted myocardium is jeopardized by hypoxia, inflammation, or oxidative stress. Liu et al. engineered BM-MSC to overexpress angiogenin [107], which improved hypoxic resistance in culture and was translated into an increase in cell engraftment and functional and histological recovery induction. Cell overexpression of hemeoxygenase-1 through adenoviral transfection showed superior therapeutic capacity, mainly through protection from inflammation and apoptosis [108], whereas targeted Akt overproduction in MSC restored cardiac function 2 weeks after MI through paracrine actions, including protection from hypoxia-induced apoptosis, release of cytokines, and preservation of tissue metabolism [109–111]. Others have explored antioxidants, like Song et al. who published that reactive oxygen species (ROS) diminished BM-MSC adherence to the substrate, but when treated with an ROS scavenger (N-acetyl-L-cysteine), engraftment was improved and the increase in fibrosis and infarct size prevented [112].

Hsp20 overexpression also protected MSC from oxidative stress and improved their beneficial activities [97].

However, viral or genetic modification of cells implies certain risks that currently make it difficult for a devised therapy to reach the bedside. Bioengineering uses biocompatible materials to improve or direct cell therapy and either synthetic or naturally derived systems have been employed. Jin and coworkers seeded BM-MSC on poly(lactide-co-1-caprolactone) patches which when applied on a rat cryoinjury model were able to improve cardiac function and decrease infarct size [113]. Porcine small intestine submucosa, a decellularized substrate, has been employed to treat a rabbit model of chronic MI, showing a significant benefit upon contractility and histology, as well as cell migration towards the injured tissue [114]. The cell sheet technology allows increasing thickness through stacking of constructs, as shown by Chen et al. [115], where its transplantation in a rat syngenic model of cardiac ischemia improved cardiac function as well as paracrine secretion of therapeutic molecules by grafted cells. Dr. Mori's group compared the transplantation of a cell sheet seeded with ADSC versus fibroblasts, showing the superior effect of the mesenchymal progenitors [116]. Recently, autologous ADSC were transplanted along with allogenic ESC-derived $CD15^+$ cardiac progenitors in a monkey model of infarction, demonstrating the safety of the procedure, although the functional outcome was not analyzed [117].

Finally, a word of caution must be added. Animal models of the disease are a powerful tool to explore the feasibility of a certain therapy, as MSC treatment of MI, but despite positive and reproducible results, rodent and even large animal models are just oversimplifications of the more complex setting of the human disease. As above stated, animals where cell therapy is applied are not elderly, nor severely diseased, thus making any result, even if tremendously positive, just a clue or hint before proceeding to the final application to patients, where the real safety and effectiveness can be assessed.

6. Mesenchymal Progenitors and Clinical Application

Several clinical trials have been performed with autologous BM-MSC, proving their safety when transplanted in patients with either acute or chronic myocardial infarction [118–120]. Moreover, the first clinical trial designed as a randomized study showed an improvement in the cardiac func tion 3 months after BM-MSC intracoronary infusion in patients with acute MI [120]. In view of the encouraging results of the previous clinical trials, new phase-I/II studies have been initiated, including the transendocardial autologous cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT; http://www.clinicaltrials.org/NCT00768066/), the Prospective Randomised study Of MSC THErapy in patients Undergoing cardiac Surgery (PROMETHEUS) trial (http://www.clinicaltrials.org/NCT00587990/), and the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON) pilot study (http://www.clinicaltrials.org/NCT01087996/) [121], among others.

BM-MSCs from allogeneic origin have been tested as an off-the-shelf cell product. The first phase-I, randomized, double-blind, placebo-controlled, dose-escalation study was performed in 53 patients with acute MI, who intravenously received one of three doses of BM-MSCs (0.5, 1.6 or 5.0×10^6 BM-MSC/Kg body weight) derived from a single cell donor (Prochymal; Osiris therapeutics, Inc.) or placebo [122]. Safety of the procedure was proven, showing fewer episodes of ventricular tachycardia and even a better lung function in the cell-treated group. Also, renal, hepatic, and hematologic laboratory indexes were similar in the two groups and no patient developed tumors. Importantly, a significant increase was detected in the ejection fraction (EF) of the treated patients. In a magnetic resonance imaging substudy, cell treatment, but not placebo, increased left ventricular ejection fraction and led to a reversal of adverse remodeling after 6 months of treatment. Now, a phase-II multicentre trial of ProchymalTM has been started (http://www.clinicaltrials.org/NCT00877903/).

Furthermore, BM-MSC safety has been tested in patients with moderate-to-severe chronic heart failure in a phase-II, randomized, single-blind, placebo-controlled, dose-escalation, multicenter study. In this clinical trial, the patients received an endoventricular injection of an allogeneic BM-MSC product (Revascor, Mesoblast Ltd.) along the infarct border zone and no procedure-related complications were reported. Analysis of the data obtained after 6 months of followup (http://www.mesoblast.com/newsroom/asx-announcements/archives/) showed a significant decrease in the number of patients who developed any severe or major adverse cardiac event, such as composite of cardiac death, heart attack, or need for coronary revascularization procedures. Moreover, the first cohort in the study ($n = 20$ patients), which received the low dose of the cell treatment, showed a significantly greater increase in the EF when compared with the control group [123].

On the other hand, regarding other sources of MSC such as adipose tissue, no clinical trials have been initiated yet, despite the fact that the beneficial potential of ADSC has been preclinically demonstrated [83]. Until now, only the noncultured adipose stromal vascular fraction is being tested at the clinical level. The first study, a double-blind, placebo-controlled trial named APOLLO (http://www.clinicaltrials.org/NCT00442806/; [124]) where AMI patients received autologous adipose derived stem cells by intracoronary infusion, was proven safe. Now, a phase II/III ADVANCE trial has been initiated to evaluate their efficacy (http://www.clinicaltrials.org/NCT01216995/).

In general, the results obtained from the many clinical trials performed, either with MSC or other stem cell populations (mainly BM-derived cells and skeletal myoblasts), have taught us several important lessons that will help to design and interpret the following clinical trials. (i) Cell treatment is not equally efficacious in all the patients. In general, it seems that the worse the heart damage (meaning severely decreased postrevascularization LVEF or high degree of infarct transmurality), the better the benefit induced by the

transplanted cells seems to be [125–127]. (ii) Cell dose and timing for treatment are critical. Thus, a meta-analysis of the results obtained in the most relevant clinical trials performed in acute MI patients treated with BM cells has shown a significantly greater effect in those patients that received high cell doses (10^8 cells). Also, the same study showed a greater beneficial effect when cells are infused during the first week after the infarct [128]. (iii) Autologous treatment is not necessarily the best. Until now, most of the clinical studies have been designed for autologous cell application in order to avoid the immunorejection of the transplanted cells. However, it has to be borne in mind that stem cells derived from aged patients with risk of atherosclerosis or other diseases might be defective, and thereby, treatment with them might not be as efficacious as with cells derived from young healthy donors [129–131]. In that sense, the use of MSC, which present immunomodulatory properties [132], could be of great relevance. Thus, advantages of allogeneic MSC treatment would be that, together with the putative greater paracrine effect that allogeneic cells derived from a healthy donor could exert, a fully tested clinical grade ready to use allogeneic cell product could be available for any patient. Importantly, patients with acute MI could also be eligible for such treatment. Furthermore, the logistical complexity and manufacturing costs that autologous cell preparation implies would be significantly reduced by the allogeneic application. However, caution should be taken when taking into consideration the issues related to their immune privilege explained above.

Thus, although it is mandatory to better understand the mechanisms involved in the MSC phenotype switch and to elucidate how this could affect the cells' potential benefit, it has to be considered that, in any case, because MSC would not differentiate towards cardiovascular cells and would act as a paracrine factor source [111], their permanent presence in the heart might not be necessary for therapeutic purposes. In that case, a temporarily action should be sufficient for exerting their benefit. Phase-II clinical trials are currently assessing the efficacy of the allogeneic MSC treatment, together with the long-term safety. If allogeneicity of the cells diminishes their effectiveness, several options could be considered, like temporal patient immunosuppression and/or donor-recipient HLA-II mismatch minimizing. As a consequence, the increase in the rate of engraftment of transplanted cells is so far one of the main challenges. As already indicated, the use of scaffolds could improve this factor. Interestingly, a clinical trial has been performed in 15 patients with chronic MI who were treated with a collagen scaffold previously seeded with bone marrow mononuclear cells [133]. The cellularized patch was implanted onto the pericardium and no adverse events were reported, showing the feasibility and safety of the treatment. Furthermore, a limiting effect in ventricular wall remodeling and an improved diastolic function were detected. These positive results will probably promote new larger randomized controlled trials, where mesenchymal and other stem cell populations might be tested in combination with scaffolds, thus leading to a further step in the therapeutic use of stem cells.

7. Conclusion

Mesenchymal cells have raised substantial interest in recent years due to their potential and versatility. Although we are only now starting to understand the mechanisms by which they repair or induce the repair of damaged organs, their pleiotropic activity and the technical ease of manipulation makes them good candidates for the treatment of the MI. Though waiting for randomized, double-blinded, placebo-controlled clinical trials in which large cohorts of patients could participate, the available data demonstrates the safety of the therapy and points towards a positive effect, further encouraging new investigations. The addition of the latest improvements in the field, including *in vitro* conditioning and bioengineering, will surely suppose a further step towards finding an optimized treatment. However, certain issues, mainly immunomodulatory capacity and allogenic use, need to be better understood.

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Clinical Study

Multipotent Mesenchymal Stromal Cells for the Prophylaxis of Acute Graft-versus-Host Disease—A Phase II Study

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The efficacy and the safety of the administration of multipotent mesenchymal stromal cells (MMSCs) for acute graft-versus-host disease (aGVHD) prophylaxis following allogeneic hematopoietic cell transplantation (HSCT) were studied. This prospective clinical trial was based on the random patient allocation to the following two groups receiving (1) standard GVHD prophylaxis and (2) standard GVHD prophylaxis combined with MMSCs infusion. Bone marrow MMSCs from hematopoietic stem cell donors were cultured and administered to the recipients at doses of $0.9–1.3 \times 10^6$/kg when the blood counts indicated recovery. aGVHD of stage II–IV developed in 38.9% and 5.3% of patients in group 1 and group 2, respectively, ($P = 0.002$). There were no differences in the graft rejection rates, chronic GVHD development, or infectious complications. Overall mortality was 16.7% for patients in group 1 and 5.3% for patients in group 2. The efficacy and the safety of MMSC administration for aGVHD prophylaxis were demonstrated in this study.

1. Introduction

Severe graft-versus-host disease (GVHD) is a life-threatening complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1, 2]. Steroids are the first-line treatment for established GVHD and have a response rate of 30–50%. However, the outcome for patients with severe, steroid-resistant acute GVHD is poor, and overall survival is low [3]. A large variety of drugs, such as corticosteroids, methotrexate, cyclosporine, and mycophenolate mofetil, are used for GVHD prophylaxis, but, nevertheless, approximately 20–80% of patients develop GVHD after allo-HSCT [4, 5]. Therefore, it is very important to develop new, effective methods for GVHD prevention.

Multiple immune processes underlie the condition that is clinically expressed as GVHD after allo-HSCT [6]. The recipient's antigen-presenting cells play an essential role in GVHD development. Host dendritic cells (DCs) have been identified as crucial for the priming of the CD4+ and CD8+ donor T-cells that lead to GVHD onset [7] ("direct" allorecognition), while donor DC also participate through "indirect" allorecognition [8].

Bone-marrow-derived multipotent mesenchymal stromal cells (MMSCs) are able to differentiate *in vitro* into cells of mesenchymal origin [9, 10]. MMSCs are immunosuppressive, which has been demonstrated by *in vitro* coculture experiments with allogeneic lymphocytes. These cells do not induce lymphocyte proliferation, interferon-γ production, or the upregulation of activation markers [11, 12]. Several key mechanisms have been described that contribute to the MMSCs' direct or indirect alteration of T-, NK, B- and dendritic cell function.

The development of GVHD is mainly mediated by T-cells, and MMSCs can inhibit T-cell function. MMSCs downregulate the responses of naive and memory antigen-specific T-cells to their cognate peptides, and this is an effect that is contact dependent and does not appear to be mediated by DCs [13]. MMSCs are able to attenuate T-cell production

of IL-2, which results in decreased formation of cytotoxic CD8+ T-cells [11] and directly inhibits NK cell proliferation and cytotoxic activity [14]. MMSCs cause the arrest of T-cell division, but they have no effect on early activation [15]. MMSCs induce apoptosis in activated T-cells but have no effect on resting T-cell proliferation [16]. Moreover, MMSCs promote the formation of Th1 and Th3 regulatory T-cells as well as IL-10 production, which both prevent GVHD development [17]. Studies of the interaction between MMSCs and B-cells have demonstrated that MMSCs can inhibit B-cell proliferation, differentiation, and chemotaxis [18, 19]. It is worth noting that MMSCs inhibit the production of antibodies, which makes MMSCs useful for treating autoimmune diseases, such as diabetes, arthritis, multiple sclerosis, and Crohn's disease [20].

MMSCs affect DCs, and this can alter their role as mediators of GVHD. MMSCs are capable of blocking the differentiation of monocytes and bone marrow precursors into DCs [21–23] and inhibiting the upregulation of CD1a, CD40, CD80, CD86, and HLA-DR expression during DC maturation, which maintains DCs in an immature state [24]. Moreover, MMSCs downregulate the secretion of the Th1-promoting cytokine IL-12 [24]. The generation of regulatory DCs may be mediated by soluble factors such as IL6 and prostaglandin E2 [25–27]. MMSCs also produce the "tolerogenic" cytokine IL-10 [28]. Thus, MMSCs help to prevent GVHD.

The ability of MMSCs to inhibit the development of GVHD requires not only cell-contact-dependent signals but also contact-independent signals, including prostaglandin E_2, IL-6, IL-10, indoleamine 2,3-dioxygenase (IDO1), and transforming growth factor-β [28–31]. Of these, IDO1 in particular has been identified as a key mediator of MMSCs-based immunosuppression [32–34]. MMSCs inhibit complement activation by their production of factor H, and this may be an additional mechanism underlying the broad immunosuppressive capabilities of MMSCs [35].

Thus, there is sufficient *in vitro* evidence to support the use of MMSCs in the prevention and treatment of GVHD. Furthermore, a number of patient cohorts treated with MMSCs have been reported, and the results have been promising to date [36, 37]. No patients have had side effects during or immediately after the infusions of MMSCs [38].

It has been shown that umbilical cord blood-derived MMSCs were very effective for GVHD prevention but not for treatment in the xenogenic model of NOD/SCID mice [39, 40].

However, there are no clear published data regarding the preferred dose, the timing, and the frequency of MMSC infusion. A phase III, randomized controlled trial on the use of MMSCs in acute GVHD in humans is currently underway, and the first results are promising [41]. Importantly, neither acute nor long-term adverse events have been reported following the infusion of MMSCs, so it is possible to use these cells for aGVHD prevention.

The aim of this study was to investigate the safety and the efficacy of MMSC administration for GVHD prophylaxis. The randomized, prospective clinical trial was approved by the local ethics committee and was begun in October 2008. It was based on the random allocation of patients to the following two groups: (1) the group receiving the standard GVHD prophylaxis and (2) the group receiving the standard GVHD prophylaxis combined with the infusion of the hematopoietic stem cell donors' MMSCs. The data obtained demonstrated a significantly reduced development of aGVHD in patients who received MMSCs.

2. Materials and Methods

2.1. Patients. Thirty-seven patients who had received allo-HSCT from related donors were eligible for the study between October 2008 and May 2011. They were randomly allocated to the following two groups: (1) a group receiving the standard GVHD prophylaxis and (2) a group receiving the same prophylaxis combined with MMSC infusion. For each case, the MMSCs were derived from the corresponding hematopoietic stem cell donor. The patients' characteristics are presented in Table 1. All work was conducted in accordance with the Declaration of Helsinki (1964). This study was approved by the local ethics committee, and the donors and patients provided written informed consent.

2.2. Procedures and Definitions. The patients received either myeloablative or reduced-intensity conditioning (Table 1). Conditioning was myeloablative in 27 patients and included cyclophosphamide (60 mg/kg/day for 2 days) combined mainly with busulfan (4 mg/kg/day for 4 days). Ten patients had low-intensity conditioning regimens with either fludarabine phosphate (30 mg/m^2/day for 6 days) combined with busulfan (4 mg/kg/day for 2 days) and antithymocitic globulin (ATG) (10 mg/kg/day for 4 days) or fludarabine phosphate (30 mg/m^2/day for 5 days) combined with BCNU (200 mg/m^2/day for 2 days), melphalan (140 mg/m^2/day for 1 day), and ATG (20 mg/kg/day for 2 days).

As GVHD prophylaxis patients received cyclosporine combined with methotrexate, some patients additionally received mycophenolate mofetil or prednisolone.

Acute GVHD was graded according to internationally accepted criteria [42].

2.3. Laboratory Methods. The characteristics of the donors and the grafts are shown in Table 2.

MMSCs were derived from 25–30 mL of the stem cell donors' bone marrow. For mononuclear cells, the bone marrow was mixed with an equal volume of alfa-MEM (ICN) media containing 0.2% methylcellulose (1500 cP, Sigma-Aldrich). After 40 min, most erythrocytes and granulocytes had precipitated, while the mononuclear cells remained in suspension. The suspended (upper) fraction was aspirated and centrifuged for 10 minutes at 450 g.

The cells from the sediment were resuspended in a standard cultivation medium that was composed of alfa-MEM supplemented with 4% platelet lysate obtained from the donors' thrombocyte concentrates, as previously described [43], 2 mM L-glutamine (ICN), 100 U/mL penicillin (Ferein), and 50 μg/mL streptomycin (Ferein). The cells were cultured at 27×10^6 cells per T175 cm^2 culture flask

Table 1: Characteristics of the patients and treatments.

Group characteristics	First group (1) Standard GVHD prophylaxis	Second group (2) Standard GVHD prophylaxis + MMSCs
Sex of patient, male/female	7/11	8/11
Median age, years (range)	29 (19–60)	34 (20–63)
Diagnosis, *n*		
AML/MDS	10	14
ALL	4	2
CML	3	3
CLL	1	
Disease stage, *n*		
complete remission	15	19
non-complete remission	3	0
Conditioning regimen, *n*		
RIC	4	6
MAC	14	13
Observation time, months	3.5–30.5	2.5–32

AML: acute myeloid leukemia, MDS: myelodysplastic syndrome, ALL: acute lymphoid leukemia, CML: chronic myeloid leukemia, CLL: chronic lymphoid leukemia, RIC: reduced intensity conditioning, MAC: myeloablative conditioning.

Table 2: MMSC donor and graft characteristics.

Donors	Values
Sex of donors, M/F	19/18
Median age, years (range)	34 (13–68)
MMSCs	
Culture passage at MMSCs harvest	0–3
Immunophenotype	
CD105 (Endoglin)	98,6 ± 0,2%
CD73 (SH3, SH4)	98,1 ± 0,5%
CD90 (Thy-1)	98 ± 0,5%
CD59	98,8 ± 0,1%
Fibroblast Surface Protein (FSP)	97 ± 0,4%
CD31 (PECAM-1)	2,5 ± 0,7%
HLA-DR	3,7 ± 0,7%
CD34	0,00%
CD45	4,5 ± 0,8%
CD14	2,0 ± 0,6%
Proportion of viable cells, %	95.3 ± 1.3%
Median MMSCs cell dose ($\times 10^6$/kg, range)	1.1 (0.9–1.3)
Relative expression level of several genes in MMSCs on passage 2	
IL-6	2.57 ± 0.98
Ptges	10.07 ± 3.16
CSF1	2.04 ± 0.39
IDO1	0.36 ± 0.132
IL-10	2.73 ± 0.6
CFH	1.98 ± 0.36

(Corning-Costar). When a confluent monolayer of cells had formed, the cells were washed with 0.02% EDTA (ICN) in a physiologic solution (Sigma-Aldrich) and then trypsinised (ICN). The cells were seeded at 4×10^3 cells per cm^2 of flask area. The cultures were maintained in a hypoxic atmosphere at 37°C in 5% CO_2 and 5% O_2. The number of harvested cells was counted directly; cell viability was checked by trypan blue dye exclusion staining. MMSCs were harvested in 6% polyglucin (public corporation Biochimik) and were either cryopreserved in 10% dimethyl sulphoxide (ROTH) or resuspended at a final concentration of $3–7 \times 10^6$ cells per mL polyglucin, according to local guidelines, and infused intravenously into the patient at target dose 10^6 per kg of body weight.

All MMSCs were immunophenotyped with following markers: CD105, CD73, CD45, CD34, CD14, and HLA-DR using standard protocols. Antibodies were purchased at BD Pharmingen (CD105, CD59, CD73, CD90, CD31, CD34, and CD14), Sigma (CD45, FSP), and DAKO (HLA-DR).

Total RNA was extracted from MMSCs by the standard method [44] and cDNA was synthesized using oligo(dT) primers. The gene expression level was quantified by real-time quantitative PCR using hydrolysis probes (Taqman) and ABI Prism 7000 (Applied Biosystems). Gene-specific primers were designed by the authors and synthesized by Syntol R&D. All primers and probes could be provided upon request. The relative gene expression level was determined by normalizing the expression of each target gene to that of β-actin and GAPDH and was calculated using the $\Delta\Delta C_t$ method [45] for each MMSCs sample.

The criteria for the admission of MMSCs for clinical use included a spindle-shape morphology, the absence of visible clumps or contamination by pathogens, standard immune phenotyping [46] for the expression of surface molecules [47] and data on the *in vitro* differentiation of the cells into osteoblasts or adipocytes [48]. The cells were given as intravenous infusions when the blood counts were indicative of recovery following allo-HSCT (more than 1×10^9/L

leukocytes). The MMSC dose varied from 0.9 to 1.3 $\times$ 10^6/kg. Cells for 7 of the infusions were harvested fresh from cultures and were given to the patients. For the other 11 cases, frozen cells were thawed and infused.

2.4. Statistical Analysis. Data were analyzed using Student's *t*-test, with the last data collection in June 2011.

3. Results and Discussion

The MMSCs were expanded using the platelet lysate obtained from the donors' thrombocyte concentrates to avoid the transmission of zoonoses and the immune reactions possible if fetal calf serum were used [49]. All of the human components used for MMSC cultivation were from hematopoietic stem cell donors. It was assumed that MMSCs were transplantable across major histocompatibility complex class 1 barriers [41, 50]. However, it was recently shown that MMSCs are weakly immunogenic *in vivo* when transplanted across major histocompatibility complex class 1 barriers [51]. Thus, only MMSCs derived from the stem cell donors were used in this trial. The MMSC characteristics are presented in Table 2. Nineteen patients received MMSCs for GVHD prophylaxis. The exact date of infusion of MMSCs after transplantation, the MMSCs dose, and the exact pharmacologic immunosuppression applied in each patient are presented in the Table 3.

MMSCs were administered when the blood counts were indicative of leukocytes' recovery (leukocytes more than 1 $\times$ 10^9 per liter). The time of administration was chosen at the time of graft activation and thus also at the time of GVHD manifestation. The median day of administration was day +28 after HSCT (19–54 days). Most of the patients had moderate fever and chills for 24 hours after MMSC administration, but there were no other complications.

In the group receiving the MMSCs, acute GVHD of grade II developed in only one case (5.3%) (Tables 3 and 4). This case was a 59-year-old patient with CML, who had received a transplant in the 1st chronic phase from an HLA identical related donor. The blood counts were recovered at day +17 after allo-HSCT. The acute GVHD manifested at day +25 with skin involvement prior to MMSC injection. The MMSCs were administered only at day +30, as the required cell dose was not ready at day +17 due to the slow growth of the donor's MMSCs. The GVHD prophylaxis included cyclosporin, methotrexate, and prednisolone. The hematopoietic stem cell donor was 56-year-old, and his MMSCs grew slower than the MMSCs from other donors. Moreover, the relative expression level of the immunomodulatory factors expressed by his MMSCs was altered compared with the others (Table 2), the IL-6 level increased 2.7-fold, and the CSF1 level increased 1.8-fold, while the expression level of IL-10 decreased 1.7-fold, the CFH level decreased 12-fold, and the Ptges level decreased 11-fold. It is possible that the increased level of IL-6 led to the activation of the donor T-cells and B-cells [52]. Additionally, the increased level of CSF1 in the donors' MMSCs could have further enhanced macrophage activation, which would result in GVHD progression instead of inhibition. Moreover, the decreased production of factors that inhibit GVHD [26, 28–30] by MMSCs from this donor did not permit GVHD prevention. However, this single case of ineffective prophylaxis did not allow clear conclusions to be made about the significance of these factors expressed by MMSCs *in vitro* in the efficiency of GVHD prophylaxis. Nevertheless, clinical improvement was registered following MMSCs infusion, but, in one month, GVHD progression to grades III-IV and involving the skin, gut, and liver occurred.

In the control group, 6 out of 19 patients had acute GVHD of grades II–IV (33.3%), which corresponded to the data from other investigators [2]. The outcomes of patients in each group are depicted in Table 4.

Though the groups of patients are not great, yet there is a significant difference in the development of acute GVHD in patients who received MMSCs prophylaxis compared with the control group ($P = 0.009$). Despite the high statistical differences between these groups, the data could not provide solid evidence for the efficacy of the approach due to limited number of patients included in the trial. MMSC injection did not influence the development of chronic GVHD (Tables 3 and 4). The diagnosis of chronic GVHD is usually made earlier than 100 days after allo-HSCT [53]. The MMSCs injected at 28 days after allo-HSCT have only a small influence on chronic GVHD development likely due to their short life span and improper homing in the host [54, 55]. Clinical studies have shown that patients who develop GVHD have a lower risk of relapse [56]; moreover, it was shown that cotransplantation of mesenchymal stromal cells and hematopoietic stem cells may prevent GVHD, but the relapse rate was obviously higher than the control group [57], although we found no difference in the relapse rates of both groups of patients. It deserves to note that in this study MMSCs were not cotransplanted with hematopoietic stem cells but infused after transplant activation.

There were no differences in the graft rejection rates or the infectious complications. The overall mortality was 22.2% in the standard prophylaxis group and 5.3% in the MMSC-treated group.

4. Conclusions

The current study is the first clinical trial to evaluate the feasibility and the safety of platelet lysate *in vitro* expanded stem-cell donor MMSCs for the prevention of acute GVHD. A high efficacy of MMSCs in GVHD prophylaxis was clearly demonstrated even on such limited number of patients, and no adverse events could be directly attributed to MMSC administration. In order to make a MMSC administration in the prevention of acute GVHD a candidate for inclusion in the standard protocols for GVHD prophylaxis, further investigations on the enlarged groups of patients should be performed. The data obtained support the development of new trials focused on the use of this approach in haploidentical and unrelated HSCT.

Table 3: Patients treatment.

Patient	Age	Diagnosis	Conditioning regimen	MMSCs infusion				GVHD prophylaxis	GVHD stage (days after allo-HSCT)	Chronic GVHD
				Days after allo-HSCT	Passage number (P)	Dose per kg	Cryopreservation			
First group (1) standard GVHD prophylaxis										
KO	38	CML	MAC					CSA + Mtx + pr	II (15)	Yes
IV	27	ALL	MAC					CSA + Mtx+	II (62)	Yes
TB	19	AML	MAC					CSA + Mtx+	II(20)	Yes
CT	59	AML	RIC1					CSA + Mtx + MM	IIII–IV (100)	Yes
SV	25	AML	MAC					CSA + Mtx+	No	No
ZL	34	CML	MAC					CSA + Mtx + pr	No	No
RA	19	ALL	MAC					CSA + Mtx+	I (23)	No
KL	38	MDS	MAC					CSA + Mtx+	No	No
AD	38	AML	MAC					CSA + Mtx + MM + pr	No	No
SO	24	AML	MAC					CSA + Mtx+	I (26)	No
SE	51	AML	RIC2					CSA + Mtx+	II (39)	Yes
SI	20	AML	MAC					CSA + Mtx	II (10)	Yes
ZC	24	CML	MAC					CSA + Mtx	No	No
RA	31	AML	MAC					CSA + Mtx	I (36)	No
GN	60	AML	RIC2					CSA + Mtx	No	No
GJ	24	MDS	RIC2					CSA + Mtx	No	No
SN	36	ALL	MAC					CSA + Mtx	I (19)	No
PE	22	CLL	MAC					CSA + Mtx	No	No
Second group (2) standard GVHD prophylaxis + MMSCs										
AN	34	AML	MAC	+31	P2	1	No	CSA + Mtx	No	Yes
BT	20	CML	MAC	+28	P1	1,25	Yes	CSA + Mtx + pr	No	No
KA	22	ALL	MAC	+29	P1	1,1	Yes	CSA + Mtx	No	No
PS	29	AML	MAC	+31	P0 + P1	1	No	CSA + Mtx	No	No
PN	46	AML	MAC	+54	P3	1,08	Yes	CSA + Mtx	No	No
KS	37	MDS	RIC1	+28	P1	1,1	No	CSA + Mtx + MM	I (21)	No
SE	54	AML	RIC1	+50	P3	1,05	No	CSA + Mtx + MM	No	No
RS	47	MDS	RIC1	+34	P1	0,93	Yes	CSA + Mtx + MM	No	Yes
CA	44	AML	MAC	+32	P1	1,18	Yes	CSA + Mtx	No	No
TM	28	AML	MAC	+28	P2	1,05	Yes	CSA + Mtx	No	No
IL	63	AML	RIC1	+25	P1	0,9	No	CSA + Mtx + MM	No	No
CM	50	AML	RIC1	+26	P1	1,07	Yes	CSA + Mtx + MM	I (48)	No
BP	33	AML	MAC	+29	P1	1,15	No	CSA + Mtx	No	Yes
MK	33	AML	MAC	+22	P1 + P2	1,12	Yes	CSA + Mtx	I (17)	Yes
FE	39	CML	MAC	+24	P1 + P2	1,3	Yes	CSA + Mtx + pr	I (18)	No
TV	40	CML	MAC	+30	P1 + P2	1,26	Yes	CSA + Mtx + pr	II (25)	Yes
AI	22	ALL	MAC	+19	P0 + P1	1,25	No	CSA + Mtx	I (73)	No
DE	31	AML	RIC1	+28	P1	0,96	Yes	CSA + Mtx + MM	No	No
SS	34	AML	MAC	+24	P1 + P2	1,39	Yes	CSA + Mtx	No	No

AML: acute myeloid leukemia, MDS: myelodysplastic syndrome, ALL: acute lymphoid leukemia, CML: chronic myeloid leukemia, CLL: chronic lymphoid leukemia, RIC: reduced intensity conditioning ((1) fludarabine phosphate + busulfan + ATG, (2) fludarabine phosphate + BCNU + melphalan + ATG), MAC: myeloablative conditioning, CSA: cyclosporine, Mtx: methotrexate, pr: prednisolone, MM: mycophenolate mofetil.

Table 4: Patients' outcome.

Group characteristics	First group (1) Standard GVHD prophylaxis ($n = 18$)	Second group (2) Standard GVHD prophylaxis + MMSC ($n = 19$)
Death at +100 days, *n*, %	1 (10%)	0
aGVHD (II–IV grade), *n*, %	6 (33.3%)	1 (5.3%)
cGVHD (lim + ext), *n*, %	6/17 (35.3%)	5/18 (27.8%)
Relapse rate, *n*, %	5/18 (27.7%)	4/19 (21.1%)
Alive, *n*, %	14 (77.7%)	18 (94.7%)

cGVHD form: lim-limited, ext-extensive.

Conflict of Interests

The authors have no relevant conflict of interests to declare.

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Research Article

Multipotent Mesenchymal Stromal Stem Cell Expansion by Plating Whole Bone Marrow at a Low Cellular Density: A More Advantageous Method for Clinical Use

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Mesenchymal stem cells (MSCs) are a promising source for cell therapy due to their pluripotency and immunomodulant proprieties. As the identification of "optimal" conditions is important to identify a standard procedure for clinical use. Percoll, Ficoll and whole bone marrow directly plated were tested from the same sample as separation methods. The cells were seeded at the following densities: 100 000, 10 000, 1000, 100, 10 cells/cm^2. After reaching confluence, the cells were detached, pooled and re-plated at 1000, 500, 100, and 10 cells/cm^2. Statistical analyses were performed. Cumulative Population Doublings (PD) did not show significant differences for the separation methods and seeding densities but only for the plating density. Some small quantity samples plated in T25 flasks at plating densities of 10 and 100 cells/cm^2 did not produce any expansion. However, directly plated whole bone marrow resulted in a more advantageous method in terms of CFU-F number, cellular growth and minimal manipulation. No differences were observed in terms of gross morphology, differentiation potential or immunophenotype. These data suggest that plating whole bone marrow at a low cellular density may represent a good procedure for MSC expansion for clinical use.

1. Introduction

In recent years, a large number of studies have shown that mesenchymal stem cells (MSCs) represent an attractive option for new therapeutic approaches, due to their plasticity and differentiative potential. MSCs are multipotent stem cells that are able to differentiate into different lineages including mesodermal, ectodermal, and endodermal type cells [1–4]. MSCs can be easily isolated by their ability to adhere to plastic generating single-cell-derived colonies [5, 6] that can be expanded to obtain high numbers of cells for clinical use in cell and gene therapy for a number of human diseases [1].

Several methods have been described for isolating MSCs from bone marrow (BM), including the use of immunomagnetic beads, density gradient separation, and direct BM plating [2, 7–11]. Density gradients such as Ficoll or Percoll centrifugation are commonly used to isolate MSCs from human BM [2, 8, 11, 12] whereas direct plating is commonly used for cells from rats [10], mice [13], and rabbits [14] which have limited available BM. Hemopoietic contamination, due to the presence of macrophages, endothelial cells, and lymphocytes which also adhere to plastic, is often present in the early BM monolayer [2, 15]. However, only fibroblast-like spindle-shaped cells proliferate and form colonies termed colony forming unit-fibroblasts (CFU-Fs) which are representative of the more highly proliferative cells in MSCs [6, 16]. On the basis of isolation and expansion protocols, the CFU-Fs originate MSCs with different proliferative and differentiative potentials which may be either subtle or significant [9, 17]. The International Society for Cellular

Therapy proposed three minimal criteria to identify MSCs (an abbreviation used to indicate *multipotent mesenchymal stromal cell*): (1) the adherence to plastic; (2) the specific surface antigen expression (positivity for CD105, CD73, CD90 and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II; (3) the multipotent capacity to differentiate into osteoblasts, adipocytes, and chondrocytes under standard *in vitro* differentiating conditions [18].

The safety, feasibility, and efficiency of MSC transplantation for clinical use are currently the object of studies, and, as several protocols use extremely high numbers of MSC (until 10^9), the identification of "optimal" conditions for *in vitro* cell culture should also be investigated.

Isolation methods, including medium, plastic, seeding density, growth factors, and chemicals, influence the expansion, differentiation, and immunogenic properties of MSCs. Futhermore, donor age and disease stage [19, 20] can also influence MSC yield, proliferation rate, and differentiation potential. BM MSCs are usually isolated from BM mononuclear cells obtained after gradient separation and for their capacity to adhere to plastic.

Percoll, a suspension of colloidal silica particles, widely used at different densities to separate cells, organelles, viruses and other subcellular particles, or Ficoll a polymer of sucrose, traditionally used to separate mononuclear cells and lymphocytes, have both been used at densities of 1.073 g/mL [2, 21–23] and 1.077 g/mL [24–26], respectively, to isolate MSCs with high proliferative and differentiative potential.

In this study, using the same BM sample, we isolated MSCs from healthy donors using different separation and expansion methods. We used Ficoll, Percoll, and direct BM plating as the separating methods and tested different seeding and plating cellular densities to verify the best method to obtain a high number of MSCs for clinical use.

2. Material and Methods

2.1. Harvest and Preparation of MSCs. Bone marrow (BM) cells were harvested from the iliac crest of adult or pediatric Caucasian donors who underwent bone marrow collection for a related patient after informed consent. When available, we also used an unfiltered bone marrow collection bag (Baxter Healthcare Corporation, IL, USA) which was normally discarded before the BM infusion. The bag was washed 3 times with Phosphate Buffer Saline (PBS) 1X (Lonza, Verviers, Belgium), and the cells were collected at 200 g for 10 minutes. An aliquot of whole BM was counted and plated directly in MSC Medium (Lonza, Verviers, Belgium) containing 10% Foetal Bovine Serum (FBS) at the various densities in T25 o T75 flasks (Becton Dickinson, Franklin Lakes, NJ, USA). The remaining part of the BM sample was divided into 2 parts for the Percoll and Ficoll gradient separation. The cells were layered on a Percoll (Sigma Aldrich, St. Louis, MO, USA) gradient (1.073 g/mL density) according to a previously reported method [20] and on a Ficoll (Biochrom, Milton Road, Cambridge, UK) gradient (1.077 g/mL density). The cells were centrifuged at 1100 g for 30 minutes, and 400 g for 30 minutes respectively. The cells in the interphase were recuperated, washed twice with PBS 1X (200 g for 10 minutes), seeded in a MSC Medium containing 10% FBS and maintained at 37°C with an atmosphere of 5% CO_2 at the following densities: 100 000, 10 000, 1000, 100, and 10 cells/cm^2. After 5 days, the nonadherent cells were removed and refed every 3-4 days and when they reached confluence, they were detached, pooled, and replated for a further 3–5 passages at 1000, 500, 100, and 10 cells/cm^2.

2.2. MSC Analysis. The cells were counted and analyzed at each passage for cellular growth, viability and immunophenotype by cytofluorimetric analysis.

2.3. MSC Clonogenic and Proliferation Potential. The clonogenic potential of isolated MSCs from the 3 different BM fractions (whole BM, mononuclear cell [MNC] fraction after Ficoll and Percoll gradient) were tested by fibroblastic-colony-forming unit (CFU-F) assay. The cells were seeded at the different densities, and the medium changed every 3-4 days. MSC clonogenic precursors (CFU-F) were scored macroscopically after 2 weeks, and clusters of more than 50 cells were considered colonies. All the experiments were performed in duplicate.

On average, the CFU-Fs were counted by 2 different operators. The CFU-Fs were indicated as the fibroblastic clones obtained from the starting cellular compartment of the whole BM.

The cellular expansion growth rate of MSCs was evaluated by cell count in a Burker Chamber at each passage and expressed in terms of population doubling (PD) using the formula $\log N/\log 2$, where N is the cell number of the confluent monolayer divided by the initial number of cells seeded [20].

2.4. Cytofluorimetric Analysis of MSCs. The identification of adherent cells was performed by flow cytometry analysis. At each passage, 200 000–500 000 cells were stained for 20 minutes with anti CD105 PE (Immunostep S.L, Salamanca, Spain), CD45 FITC, CD14 PE, CD73 PE, CD44 PE, CD29 FITC (Becton Dickinson, San Jose, CA, USA), CD105 PE, CD166 FITC, CD90 FITC, CD106 PE (Beckman Coulter, Brea, CA, USA.). The labeled cells were thoroughly washed with PBS 1X and analyzed on a FACScanto II (Becton Dickinson) with the DIVA software program. The percentage of positive cells was calculated using the cells stained with Ig FITC/PE as a negative control.

2.5. Differentiation Potential Assay. For differentiation experiments, from the 1st to the 5th passages MSCs were cultured in osteogenic, adipogenic and chondrogenic medium (Lonza) according to the manufacturer's instructions. Briefly, 20 000 and 50 000 cells were plated in a T-25 flask for osteogenesis and adipogenic culture conditions, respectively, allowing the cells to adhere to the culture surface for 24 hours in MSC medium (Lonza). To induce osteogenesis and adipogenesis, the medium was replaced with specific complete induction medium (Lonza). After 21 days, osteogenic differentiation was demonstrated by the accumulation of

calcium (crystalline hydroxyapatite detection by Von Kossa staining) in separated cells plated in chamber slides in the same culture conditions.

For the adipogenic differentiation, adipogenic induction and maintenance medium were alternatively used every 3-4 days, and the presence of intracellular lipid vesicles visible after 2-3 weeks' culture was assessed by Oil Red O staining.

For chondrogenic differentiation, an aliquot of 250 000 cells was washed twice with incomplete chondrogenic medium (Lonza) in 15 mL polypropylene culture tubes. Finally, the cells were resuspended in complete chondrogenic medium, centrifuged and, without aspirating thesurnatant, the tubes were incubated at 37°C in a humidified atmosphere of 5% CO_2. Chondrogenic differentiation was due to the growth of the cells as cellular aggregates floating freely in suspension culture with Transforming Growth Factor (TGF)-beta3. The pellet was included in paraffin and stained with Alcian Blue to identify the presence of hyaluronic acid and sialomucin.

2.6. Telomere Length (TL) Evaluation. TL evaluation was carried out by Southern Blot (SB) analysis as described elsewhere [27]. Briefly, 22 μg of DNA were digested by mixing HinfI (20 U) and RsaI (20 U) (Roche Diagnostic, Mannheim, Germany) and incubating at 37 for 2 h (Figure 5). Resulting DNA fragments were then separated on a 0.8% agarose gel by electrophoresis in 1X TAE running buffer and 5 μL of ethidium bromide. Separated DNA was subsequently transferred to a positively charged nylon membrane (RocheDiagnostic Mannheim, Germany). After an overnight transfer, in order to fix DNA fragments the membrane was exposed to UV light for 10 minutes. Hybridization was carried out with the TeloTAGG Telomere Length Assay Kit (Roche Diagnostics, Manheim, Germany).

Membranes were submerged in a prehybridization solution and then incubated in the hybridization solution (2 μL of the digoxigenin (DIG)-labeled telomere-specific probe added to the prehybridization solution) for 3 h at 62°C. Then, membranes with DNA fragments linked to telomere probes were incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase (AP).

The results were visualized using AP metabolizing CDP-Star, a highly sensitive chemiluminescent substrate.

The light signal was recorded on X-ray film (Lumi-Film Chemiluminescent Detection Film, Roche Diagnostic, Mannheim, Germany) and scanned for analysis.

Median TR length was calculated using the software quantity One by Biorad (Hemel Hempstead, UK).

2.7. Statistical Analysis. Cell growth data were analyzed by SPSS 15 for Windows (SPSS Inc, Chicago, IL). The results were expressed as medians and ranges. The differences between paired samples were evaluated by Friedman's test [28, 29] and a post hoc multiple comparison analysis using the Least Significant Difference (LSD) method.

All statistical tests were two-sided and significant for a P value <0.05.

3. Results

3.1. MSC Harvest and Preparation. Ten bone marrow samples were collected from donors: 3 over 18 years of age (age range: 39–50 years) (2 male and 1 female) and 7 (all male) with ages younger than 18 years (age range: 0.5–10 years). The study was conducted according to the Helsinki Declaration. The whole BM was counted and seeded for all the experiments; the remaining part of the sample was separated into equal fractions for MSC separation by Ficoll and Percoll, respectively. The median of initial cell numbers for gradient separation was 69×10^6 (range: $21–82 \times 10^6$) and, after separation, the total recovery corresponded to 13.5% and 15.7%, respectively after Ficoll and Percoll (median value; range Ficoll: 5.0–18.9%, Percoll: 1.0–28.8%). These data showed that there were no differences between Ficoll and Percoll in terms of cell count and recovery after isolation.

3.2. MSC Isolation. Adherent cells were observed in all the samples after 3–5 days' culture and in the following 2 weeks an adherent monolayer was achieved. The BM cells rapidly generated a confluent layer of cells with an elongated, fibroblastic shape. The viability at each passage was always over 98%. No morphological differences were observed on the MSCs isolated from whole BM, Ficoll and Percoll, but when early passage cells were compared with late passage cells, MSCs showed a different morphology. The cells increased in size and showed a polygonal morphology with evident filaments in the cytoplasm especially when isolated from the adult donors.

3.3. MSC Analysis. MSCs isolated from healthy donors were analyzed for the first 3 passages with a median interval between one passage and the next of 16 days (range: 7–40). We observed heterogeneous MSC preparation and a distinct population of spindle-shaped or flat MSCs in the flasks, although no morphological differences were observed in the 3 preparations (Ficoll, Percoll, and whole BM). Figure 1 shows the clones from different methods after 10 days from seeding at the various densities. In 7 out of 10 cultures we did not observe clones in culture seedings at 10 and 100 cells. Interestingly, we observed clones at lower densities only in the 3 samples obtained from the washout of discarded BM collection bags and filters. In addition, these samples were obtained from child patients.

After detachment, the cells replated at 1000, 500, 100, and 10 cells/cm^2 formed clones that reached confluence in a median of 16 days (range: 7–40). The cells pooled and replated at the second and third passages also formed clones in all preparations. Figure 2 shows the clones at the second passage after 7 days from plating.

In order to compare the effect of 3 different separation methods and different densities on the proliferative capacity of MSCs, 3 BM samples were plated to ascertain the CFU-F frequency. The CFU-F number was calculated in relation to the initial cell number in the BM sample and by comparing the 3 separation methods. The results were

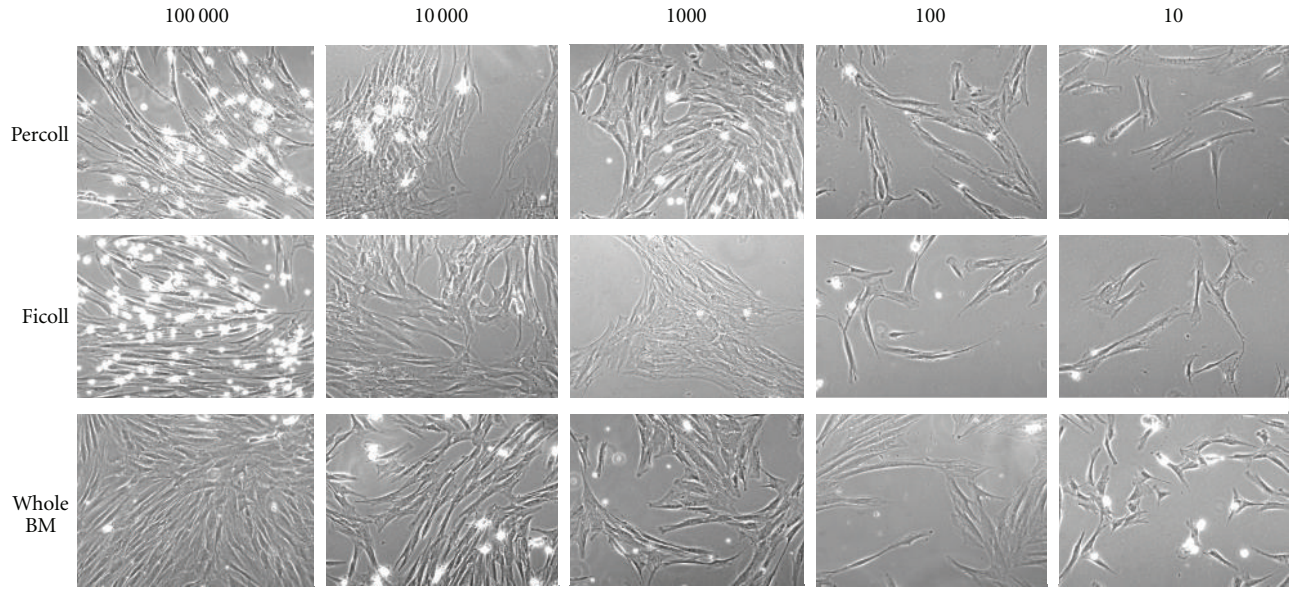


Figure 1: Phenotype of different clones observed after Percoll and Ficoll gradient separation and whole BM 10 days from seeding at different densities.

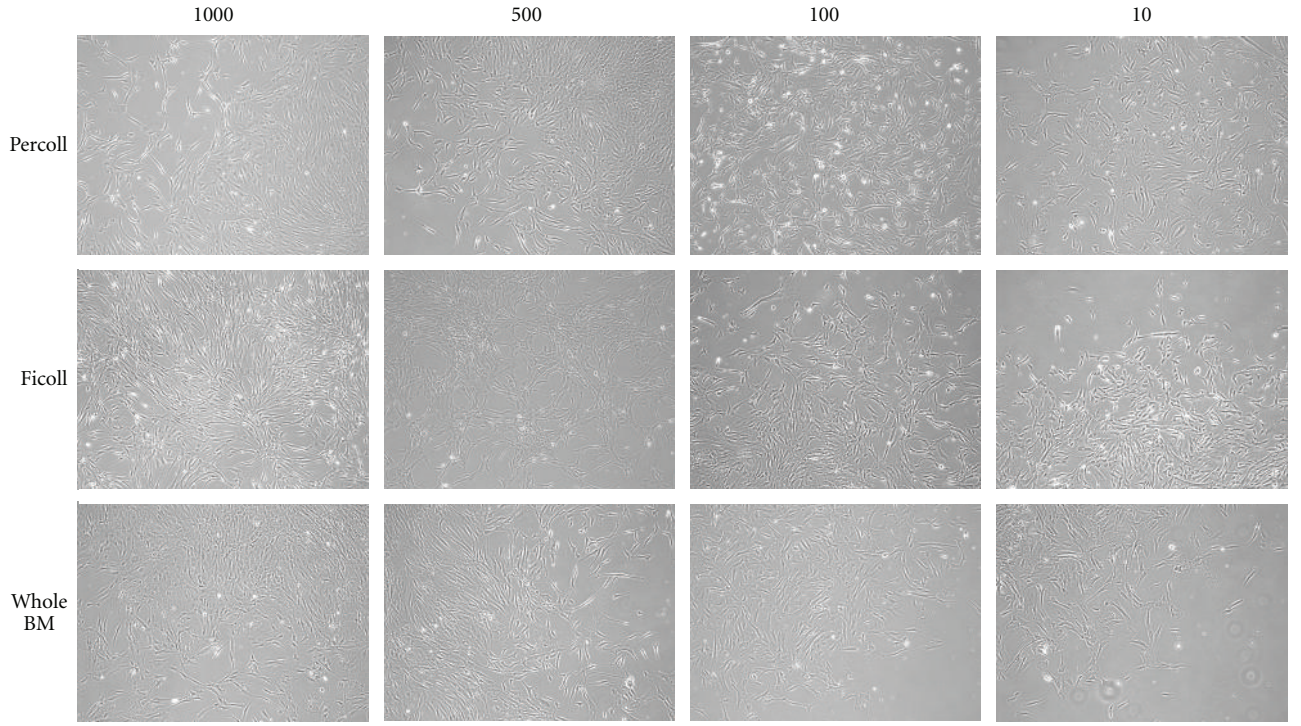


Figure 2: Phenotype of different clones observed after Percoll and Ficoll gradient separation and whole BM 7 days from plating.

as follows: whole BM showed a median of 104.4 CFU-F (range: 7.2–179.7) per 10^6 cells; after Ficoll and Percoll separation, BM showed a median of 3.8 (range: 2.7–49.8) and 0.1 (range: 0–16.4) CFU-F per 10^6. We observed no direct correlation between the number of cells plated and the number of CFU-Fs counted when analyzing the effect of the densities. In particular, at seedings of 10, 100, 1000, 10 000, and 100 000 cells/cm^2, we observed a median of 0 (range: 0-0), 0 (range: 0.0–44.4); 6.2 (range: 0.7–104.0); 62.5 (7.4–111.7) and 11.3 (3.1–13.8), respectively. Significant differences were noted among seedings at 10 with 10 000 and 100 000 cells/cm^2 between 100 with 10 000 cells/cm^2 and 1 000 with 10 000. The analysis of the 3 different separation methods and the different seeding densities, considering the

grand total of all values obtained for whole BM, Ficoll and Percoll ($N = 10$), as shown in Table 1(a), and the grand total of all values of each density, as shown in Table 1(b), did not show significantly different growth rate values at the first passage ($P = 0.49$ and $P = 0.51$, resp.).

On 4 samples, it was possible to perform a complete analysis for cumulative PD from the 1st to the 3rd passages, and the results showed that BM always showed a more advantageous growth compared to Ficoll and Percoll separation even though the statistic analysis showed no significant differences ($P = 0.653$; $P = 0.931$ and $P = 0.528$ at the 1st, 2nd and 3rd passages, resp.).

As we did not observe MSC clones in more than half of the primary cultures for the seeding at 10 and 100 cells/cm^2, these densities were excluded for the final analysis. At the 1st passage, we observed a significant statistical difference between the seedings at 1000 and 10 000 ($P = 0.028$), but not between 10 000 and 100 000 cells/cm^2.

The analysis on the effect of plating density at 10, 100, 500, and 1000 cells/cm^2 at the 1st, 2nd, and 3rd passages is summarized in Table 3. In particular, we observed significant statistical differences at the 2nd passage between plating at 10 compared to 500 and 1000 cells/cm^2 at both the 2nd and 3rd passages ($P = 0.001$ and $P = 0.017$).

3.4. Viability Evaluation. Trypan blue staining analysis showed a viability of between 98%–100% in all the analyzed samples with no differences between the two groups. The same results were confirmed after 7AAD staining in the cytofluorimetric analysis.

3.5. Immunophenotype Analysis by Flow Cytometry. During the 1st 3 passages, the cells were analyzed at each passage for the expression of CD45 and CD14, haematopoietic surface antigens; CD90; CD29, CD44; CD105; CD166 and CD106, CD73. At the 1st passage, MSCs isolated from whole BM were CD45, CD14 negative with an antigen expression less than 5% (the median was 3.0% with a range of 0.0–6.5% and 3.5% with a range of 0.0–7.0%, resp.), while they showed a high expression of CD90 (median of 90.0%, range: 65.0–93.5%), CD29 (median of 78%, range: 61.0–97.0%), CD44 (median of 83.0%, range: 65.0–99.0%) and CD105 (median of 90.0%, range: 65.0–95.0%) and a lower expression of CD106, CD166 adhesion molecules (median of 63% with a range of 2.4–88.0% and 54% with a range of 53.0–96.0%, resp.). In the MSC isolated from Ficoll and Percoll, at the 1st passage the immunophenotype showed a weak hematopoietic contamination because the median expression of CD45 was 7.5% (range: 0.0–48.0%) and 6.0% (range: 1.0–44%:), respectively, and because the expression for CD14 was 5.0% (range: 0.0–35%) and 4.0% (range: 0.0–24%), respectively, for the Ficoll and Percoll separation methods. High levels of CD90, CD29, CD44, and CD105 with values over 80% and variable percentages of CD106 and CD166 were observed without significant differences even in the cells separated by Ficoll and Percoll. In Figure 3, the median antigen expression which was analyzed at the 1st passage on the cells isolated by 3 different methods (a pool of the different seeding densities) is represented as a histogram. During the expansion time, the MSCs were negative for the hemopoietic antigen, whereas at each passage they expressed high percentages of CD90, CD73, CD29, CD44, and CD105 positive cells with the median antigen expression being over 80%.

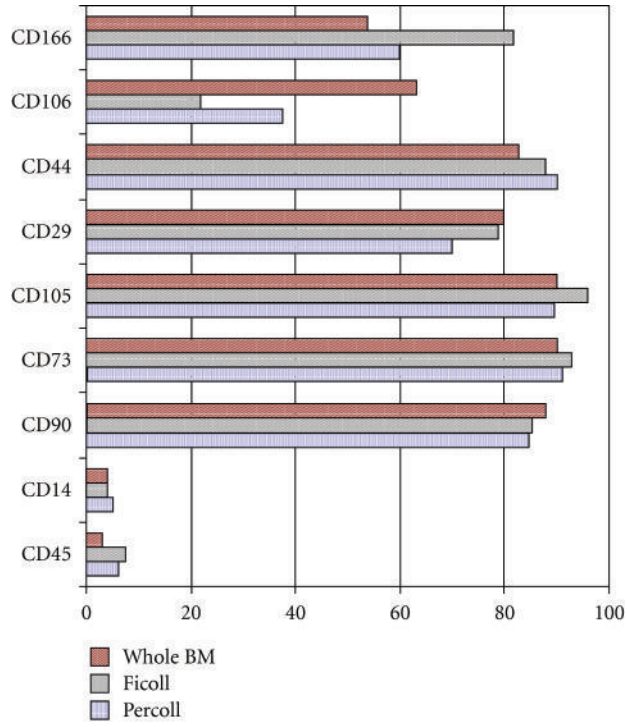


Figure 3: Immunophenotype analysis of MSCs isolated after Percoll and Ficoll gradient separation and whole BM at the 1st passage.

3.6. Differentiation Potential Assay. All samples induced into differentiation with specific medium showed a multipotential capacity because all the MSCs, independently of the separation methods and seeding and plating densities, differentiated into osteoblasts, adipocytes, and chondrocytes as shown in Figure 4. In 2 cultures of MSCs isolated from whole BM at the 1st passage, we observed the spontaneous presence of some clones of adipogenic and osteoblastic cells, respectively (data not shown).

3.7. Telomere Length Analysis. We analyzed 9 samples harvested from pediatric healthy donors of BM (male and female); the MSCs used for this analysis were all at the 3rd seeding passage and were obtained by Ficoll ($N = 3$), Percoll ($N = 3$), and direct whole BM plating ($N = 3$).

We observed a median of 11543 pb (range: 12181–11504 pb), 12906 (range: 13406–12016), and 10725 pb (range: 12060–10578 pb), respectively, for the percoll, whole BM, and Ficoll group.

4. Discussion

In recent years, a number of insights into MSC biology, as well as their immune regulatory proprieties and regenerative

Table 1: Analyses of separation methods and different density effects on seeding.

(a) Analysis of different density effects on seeding (first passage).

$N = 10$	Whole BM	Ficoll	Percoll
Median	8.56	1.76	.69
Minim	.12	.16	.00
Maxim	419.23	111.18	433.26

(b) Analysis of different density effects on seeding (first passage).

$N = 10$	10 cells/cm^2	100 cells/cm^2	1000 cells/cm^2	10 000 cells/cm^2	100 000 cells/cm^2
Median	.00	2.33	2.66	1.64	.41
Minim	.00	.00	.00	.20	.14
Maxim	531.34	205.20	29.12	5.96	5.52

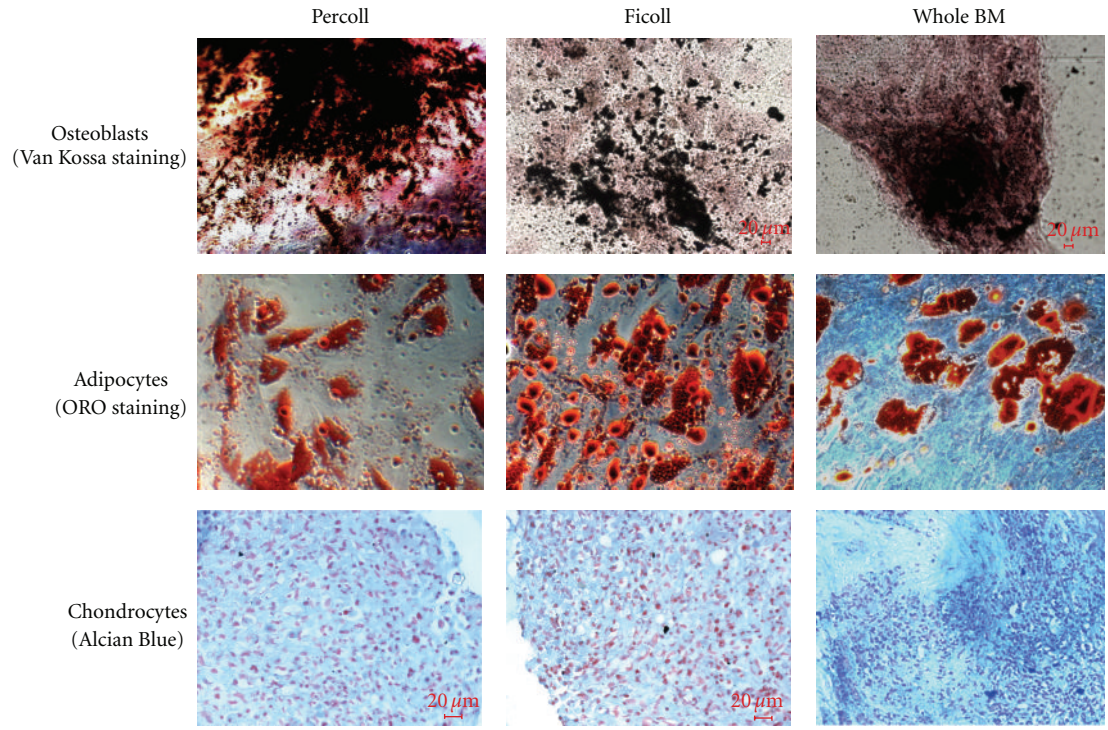


Figure 4: Differentiation of MSCs isolated after Percoll and Ficoll gradient separation and whole BM at the 3rd passage.

potential, have provided support for considering MSCs as a good candidate for cellular therapy for regenerative medicine, cancer gene therapy, and the treatment of immunologic diseases.

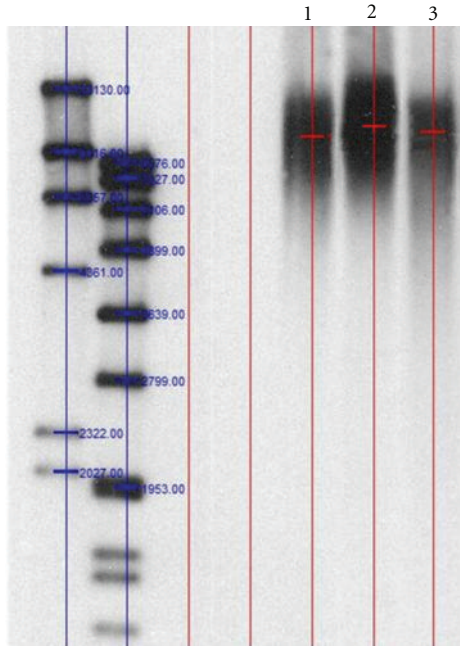
Although the culture of MSCs has been studied for over 30 years, standard criteria to isolate and characterize these multipotent stem cells have yet to be developed.

Several methods have been described to enrich BM MSCs for clinical applications. In this study, we tested, from the same BM sample, 3 different isolation methods and several different seeding and plating cellular densities. We analyzed the cellular growth, the number of CFU-Fs, the immunophenotype and differentiative potential in all isolated cultures to ascertain the optimal culture condition to isolate and expand MSCs for clinical applications.

MSCs isolated by their adherence to plastic culture surfaces have characteristic properties that have been well defined by a number of investigators [6, 30]. It is therefore difficult to compare data from different laboratories for both the different isolation and expansion methods and for the high variability of the cells inside the culture. It is often possible to note that in the cultures two morphologically distinct cells are present [6, 30, 31]: Type I cells that are spindle shaped and grow rapidly, and Type II cells that are broad and grow slowly. Moreover, in our experiments, we observed that the greater the number of passages, the higher the increase in Type II cell numbers. We also observed cells with intermediate morphologies. Other authors also demonstrated that samples of human MSCs obtained from iliac crest aspirates varied widely in their expandability in

Table 2: Analyses of separation method effects on seeding in terms of cumulative PD.

	1st passage		
$N = 4$ ($P = 0.653$)	Whole BM	Ficoll	Percoll
Median	3.11	3.12	2.62
Minim	1.99	.56	1.92
Maxim	5.53	3.89	3.84
	2nd passage		
$N = 4$ ($P = 0.931$)	Whole BM	Ficoll	Percoll
Median	4.55	2.68	5.88
Minim	11.41	7.62	8.00
Maxim	6.67	7.16	6.47
	3rd passage		
$N = 4$ ($P = 0.528$)	Whole BM	Ficoll	Percoll
Median	10.13	10.50	8.81
Minim	8.60	5.07	8.03
Maxim	15.66	11.11	12.14



(1) Ficoll
(2) Whole BM
(3) Percoll

Figure 5: Telomere Restriction Fragment (TRF) measurement by Southern Blot analysis in one representative experiment on cells isolated after Percoll and Ficoll gradient separation and whole BM at the 3rd passage.

culture [32]. The variation was not explained by either the gender or the age of the donors, nor was it explained by the number of nucleated cells in the sample, but apparently reflected a sampling variation in marrow aspirates from the iliac crest, since the variation was seen between two samples taken from the same volunteer at the same time [32]. MSCs arise from the complex architectural structures of perivascular cells that incompletely separate the marrow from capillaries [30], and the yield of MSCs apparently varies with the presence of such architectural structures in the aspirate site.

We observed no significant differences for the separation methods because MSCs isolated from Percoll, Ficoll, or whole BM showed significant differences in terms of morphology, growth rate at the 1st passage, cumulative PD, immunophenotype and differentiative potential. The descriptive analysis, however, showed major cellular growth in terms of absolute values and a minor hematopoietic contamination at the 1st passage with whole BM rather than with Percoll or Ficoll.

As the expandability of the MSC culture might be predictable, not from the initial growth rates in the 1st or 2nd passages, but on the basis of the assays for CFUs [6, 25, 33], we performed the CFU-F assays to compare the effect of 3 different separation methods in different cellular seeding densities. Considering the initial cell number in the BM sample, we obtained a much higher number of CFU-F in whole BM conditions rather than after gradient separation.

Moreover, it was interesting to note that the telomere length on MSCs isolated from whole BM was longer than MSCs isolated from Percoll and Ficoll methods. Thus, by cultivating the cells from whole BM we probably isolated more immature MSCs. This latter aspect is worthy of further research.

Even though MSC isolation and expansion by GMP manufactured gradient media have recently been reported [34], this study confirmed that directly plated BM offers a more advantageous method in terms of CFU-F number, minimal manipulation, hematopoietic contamination, and cellular growth (descriptive analysis). Our results (as has also recently been described by Capelli et al. [35] and Lucchini et al. [36]) show that whole BM separation methods represent a good procedure for MSC expansion for clinical use compared to MSCs obtained by gradient separation. To standardize a method of isolation and expansion, the most suitable cellular condition should be used for all samples. We therefore excluded seeding at 10 and 100 cells/cm^2, since we observed MSC clones in less than half of the primary cultures, whereas, at the 1st passage, we observed a significant statistical difference between the seedings at 1000 and 10 000, but not between 10 000 and 100 000 cells/cm^2. The CFU-F count was significantly higher at 10 000 cells/cm^2, therefore the use of this seeding density might prove to be the most advantageous condition. At this seeding density, a 10 mL BM sample, which contains approximately 100 million WBC, would require 10 000 cm^2 or 16 T630 cm^2 Cell Factories (flasks used for large scale cell culture). The time to confluence of these cultures would be 2 to 6 weeks which would require only media changes and might offer, at the first passage, the availability of cellular products (about 160 million cells) for clinical use. The procedure proposed would provide a high number of cells starting from a small quantity of BM. A

Table 3: Analyses of seeding density effects in terms of cumulative PD.

	1st passage			
$N = 4$ ($P = 0.068$)	10 cells/cm^2	100 cells/cm^2	500 cells/cm^2	1000 cells/cm^2
Median	5.28	3.27	2.08	2.28
Minim	2.49	.68	.58	1.39
Maxim	5.57	4.65	3.07	2.63
	2nd passage			
$N = 4$ ($P = 0.001$)*	10 cells/cm^2	100 cells/cm^2	500 cells/cm^2	1000 cells/cm^2
Median	10.45	6.82	4.48	4.48
Minim	8.33	4.76	2.90	2.88
Maxim	12.06	10.15	6.99	5.94
	3rd passage			
$N = 4$ ($P = 0.017$)*	10 cells/cm^2	100 cells/cm^2	500 cells/cm^2	1000 cells/cm^2
Median	14.85	9.89	8.14	6.37
Minim	13.41	6.78	3.82	4.92
Maxim	18.78	13.69	9.84	8.52

patient would therefore only undergo a BM biopsy and not an invasive procedure such as BM collection.

The results on cellular growth in terms of cumulative PD confirmed other authors' data that a low plating density results in higher yields and a faster expansion of MSCs [6, 19, 24, 33, 37]. We observed that small spindle-shaped cells in some cultures grew more rapidly at a low plating density. If however, MSCs isolated at the 1st passage were mostly broad and uneven, the expansion was slower and the cells were senescent at a low plating density. An other aspect of MSC *in vitro* aging was observed when the cells were plated at lower densities (10,100) after 4-5 passages (data not shown).

Moreover, we observed clones at lower densities only in the 3 samples obtained from the washout of discarded bone marrow collection bags and filters. These results confirmed those explained by Capelli et al., that is, filtration results in preferential trapping in the filters of hMSC precursors with good proliferative potential, with the consequent enrichment of these cells in the washouts compared with BM.

In conclusion, we observed that, in agreement with other groups with wide experience in this field, [33, 38–40], MSC populations have a diverse repertoire of distinct subpopulations, whose proliferative, immunological, and biological proprieties remain indeterminate. Phinney [40] demonstrated the biochemical heterogeneity of these subpopulations, rather than their stem-like character, contribute more significantly to the therapeutic potential of MSCs.

To our knowledge, this is the first comparative study of different isolation and expansion methods with or without gradient separation. Therefore, the plating of whole BM at a low cellular density may represent a more advantageous procedure for MSC expansion for clinical use compared to MSCs obtained by gradient separation.

Conflict of Interests

The authors indicate no potential conflicts of interests.

Acknowledgments

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