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Research article

Safety study of autologous adult bone marrow derived mesenchymal stromal cells in idiopathic pulmonary fibrosis - Pilot data

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ABSTRACT

Background: Lung transplantation is the choice of therapy in severe cases of idiopathic pulmonary fibrosis (IPF) but is compounded with post-transplant complications. The paucity of deceased organ donations underlines the need for alternate approaches that improves the quality of life. Herein, we attempted to develop an autologous adult bone marrow derived mesenchymal stromal cell (BMSC) therapy via central line access, and evaluated the safety of a single dose ($\sim 13 \times 10^6$ cells/mL), in treating "no option" IPF.

Method: The study included severe IPF subjects (n = 6) both male and female, aged 40–70 years of age with a forced vital capacity < 50%, diffusing capacity of lung for carbon monoxide (DLco) < 35% of predicted, and/or oxygen (SpO$_2$) saturation < 88% on 6 min walk distance (6 MWD). BMSCs at passage 2 were suspended in 30.0 mL normal saline and dispensed through the central line route in a respiratory intensive care unit of Gleneagles Global Hospitals. The subjects were monitored for the first 24 h for serious adverse events and hemodynamic parameters. They were followed up periodically at intervals of 1, 4, and 9 months for safety and monitoring of adverse events, including secondary objectives of changes in pulmonary function test, DLco, 6 MWD, and quality of life as per the study protocol.

Results: It was observed that central line infusions were well tolerated by all subjects. Furthermore, there was an improved quality of life.

Conclusions: BMSC central line infusion in "no option" IPF cases provided an insight into the strategies in improving the quality of life for patient and thereby increasing the therapeutic window period for lung transplantation.

1. Introduction

Treatment of idiopathic pulmonary fibrosis (IPF), one of the major causes of end-stage lung diseases, requires the use of immune suppressants and cortico-steroid therapy for prolonged periods of time. Recent expert guidelines recommend the use of only Pirfenidone, an anti-fibrotic agent, and Nintedanib, a tyrosine kinase inhibitor. Corticosteroids and immunosuppressants are no longer recommended for long-term management [1]. Exacerbations often lead to increased hospital admissions and a major setback to their quality of life (QoL). This "unmet medical need" warrants alternate approaches that are safe, effective, and increase the window period for lung transplantation. The mortality rate in such patients is high, due to shortness of breath,

difficulty in mobility, and poor survival rates. The recurrent incidents of lung damage result in scarring and fibrosis. Ultimately, this leads to the loss of lung architecture with worsening of the disease condition with an unknown etiology [2].

Adult human mesenchymal stromal cells (MSCs), which inhabit all vascularized tissues and organs, have demonstrated anti-fibrotic and immunomodulatory properties, along with regenerating damaged lung epithelium, tissue repair. Several clinical trials have been attempted using mesenchymal stromal cells which have the ability to differentiate into adipocytes, chondrocytes, and osteocytes. MSCs are characterized based on marker analysis showing negative expression of human leukocyte antigen (HLA–DR) of MHC class II, rendering these cells less immunogenic and less capable of suppressing lymphocyte reactivity in

a mixed lymphocyte reaction, thereby reducing inflammation in vivo. MSCs play analogous roles in different tissues and organs for indistinguishable stromal cell types. In the lungs, the resident type 2 alveolar stem cells signal a cross talk with infused MSCs during alveolar repair and enhance the epithelial progenitor cells aiding in tissue repair [3–5]. The potential of MSCs can be harnessed to address this unmet clinical

for Stem Cell Research (IC-SCR) and written informed and high-risk video consent was obtained prior to subject enrolment and performance of any study-related procedures (GMERF/CT/SAC/IEC/lC-SCR 2014/06/R3). This clinical trial was registered under ctri.gov.in with registration number CTRI/2015/02/005569. (Gleneagles Global Hospitals).
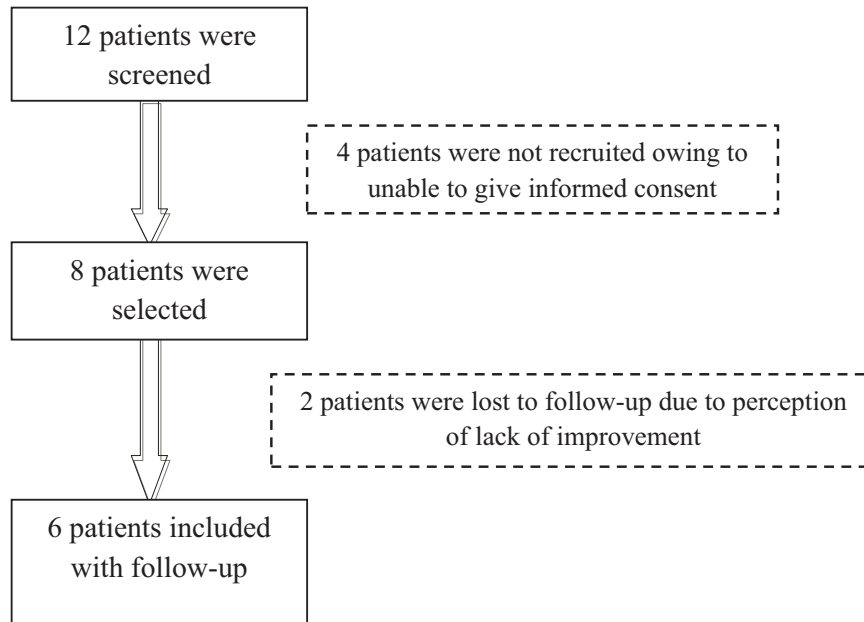
need using autologous MSCs with the primary objective of determining safety in treating patients with IPF using central line infusion and a secondary end point assessment of efficacy based on the pulmonary function test (PFT), exercise capability, and QoL measures. Thus, the use of autologous mesenchymal stromal cells derived from bone marrow (BMSCs) in the regeneration of damaged lung epithelium in IPF appears to be an attractive option owing to its hypo-immunogenic and regenerative properties.

The route of administration has been highly debated for its effects on therapeutic efficacy. Central line infusion was chosen for the current study over other cell delivery routes such as intravenous and intra-tracheal, for direct homing of BMSCs to alveolar capillaries. Central line catheterization is a simple, safe, bedside procedure with easy vein access for critically ill and morbid patients, although there are reports suggesting high rates of infection. This route is also preferred in case of resuscitation in patients where there is the potential for adverse events so that they can be treated with less chances of thrombophlebitis [6]. Autologous cell-based therapeutic models essentially emphasizes in maximizing therapeutic benefit through the choice of an appropriate cell delivery route owing to confounding factors of cell yield due to individual variations, factors of age, genetic variations in increased expansions in cell cultures, and the ease of harvesting primary tissue.

2. Material and method

2.1. Patient screening and selection

Male and female patients aged 40–70 years old (n = 6), with a diagnosis of IPF based on American Thoracic Society (ATS)/European Respiratory Society guidelines were considered for subject enrolment [7]. They were categorized for having mild to moderate disease using various estimated functional parameters. The study was approved by the Institutional Ethics Committee (IEC) and Institutional Committee

Pre-screening of patients included routine blood chemistry, chest x-ray, liver function test (LFT), serum creatinine, blood urea nitrogen, electrolytes, viral screening for HBsAg, HIV and HCV, abdominal ultrasound, echocardiography, a high resolution computerized tomography (HRCT) scan of chest, PFT, DLco, 6 MWD, quality of Life (QoL) (Saint George's Research Questionnaire-SGRQ and cough assessment test-CAT), arterial blood gas (ABG), and a thyroid profile.

The study type is interventional, single arm, open label with safety as the primary endpoint and efficacy as secondary endpoint. All the subjects were given a single infusion of autologous BMSCs ($\sim 13 \times 10^6$ cells/mL). They were followed up periodically at intervals of 1 m, 4 m, and 9 m for safety and monitoring of adverse events, including secondary objectives of changes in pulmonary function test (PFT), DLco, 6 MWD and QoL (Table 1).

2.2. Isolation and characterization of MSC phenotype

Bone marrow (~40.0 mL) from the iliac crest was harvested under aseptic conditions with anesthetic support and collected in a blood bag containing 6% acid citrate dextrose. It was diluted with sterile normal saline at a 1:1 ratio and subsequently layered over clinical grade Ficoll at a ratio of 1:3. Mononuclear cells (MNCs) were isolated through gradient centrifugation at $400 \times g$ for 30 min at 27 °C. The isolated MNCs were suspended in xenogenic free Dulbecco's minimum essential medium (DMEM) (Life Technologies, USA) with supplementation of growth factors and antibiotics as per manufacturer's recommendations. The cells were then seeded at a concentration of 1×10^5 cells/cm^2 with a change in media at regular intervals of 3 days to obtain a homogenous MSC population. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Trypsinization was performed upon reaching 70% confluent growth with 0.05% trypsin (Sigma, USA) and 0.53 mM ethylenediamine tetra acetic acid (EDTA) (Sigma, USA) [10,11]. Adherent BMSCs after the second passage to a cell dose of 13×10^6 cells/mL were segregated, washed to remove excess media,

Table 1
Detailed inclusion and exclusion criteria [8,9].

Inclusion criteria	Exclusion criteria
Confirmed diagnosis of IPF clinically and radiologically	Patient on ventilator
Male or female, aged 40–70 years old	Diagnosis of any malignancy
Disabling dyspnea at rest, poorly responsive or unresponsive to bronchodilators, resulting in decreased functional capacity	History of severe coronary artery disease (CAD), significant liver kidney disease or psychiatric illness
Post bronchodilator FEV1 $\leq 30\%$ and FVC < 30% of predicted normal values	Active smoker
DLco < 35% of the predicted value as measured by the single breath method (NDD, Switzerland)	Consent not given
Hypoxemia at rest on room air, as evidenced by $PaO_2 \leq 55$ mmHg; or oxygen saturation (SO_2) $\leq 88\%$ at rest or on 6 MWD	Breastfeeding, pregnant, or intends to become pregnant during the study
Hypercapnia, as evidenced by $pCO_2 \geq 50$ mmHg	Diagnosed case of HIV, active TB, secondary infections
Progression of end-stage pulmonary disease, as evidenced by increasing visits to the emergency department or hospitalizations for pulmonary infections and/or respiratory failure	Positive thyroid profile tests
Patients on non-invasive ventilation (NIV)	
Pulmonary hypertension as measured by 2D echo by the cardiologist	
Cessation of smoking for more than 6 months	

and suspended in 30 mL normal saline and were infused through central line as a single dose. During the entire procedure, care was taken to maintain a slow infusion rate of 30 mL/30 min.

2.3. Viability testing and immuno-phenotyping of BMSC

After trypsinization of MSCs, their viability was determined with 7-amino-actinomycin-D (7-AAD) and used only if they were in the range of above 80% viability. Cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies. Labeled cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA, USA) using the CELLQUEST software (Becton Dickinson, San Jose, CA, USA). The anti-human antibodies used were CD45-FITC, CD34-PE, HLA-DR-FITC, CD73-APC and CD90-FITC.

2.4. Osteogenic, chondrogenic, and adipogenic differentiation

Differentiation was performed to evaluate BMSC's capability to ectoderm, endoderm and mesoderm lineages as a quality control check. To induce differentiation, approximately 2×10^5 cells were placed in 15 mL polypropylene tube and centrifuged at $500 \times g$, 5 min, at room temperature to form a pellet. Then, the pellet was treated with respective differentiation media. Cells were cultured with the commercial osteogenic medium, chondrogenic medium and adipogenic medium for their differentiation to osteocytes, chondrocytes and adipocytes respectively. Medium was changed twice a week. This tri-lineage potential of MSCs was illustrated through microscopic images of Von Kossa and Oil-Red-O staining to confirm osteogenic and adipogenic differentiation of plastic adherent bone marrow-derived cell populations. Chondrogenic differentiation was confirmed by Safranin O (orange–red stain) on cell culture pellet sections.

2.5. Clinical, functional and radiological assessment

Arterial blood gases (ABGs) coupled with clinical, health-related quality of life (Saint George's Research Questionnaire-SGRQ and cough assessment test-CAT) and functional (6-min walking distance-MWD, FVC and DLCO) assessment was performed as per the protocol after infusion until the end of follow-up period (9 months) and HRCT evaluation once in 6 months after the infusion in order to estimate any potential clinical, functional and radiological differences compared to baseline.

2.6. Primary end-points (safety and toxicity levels)

In particular, patients were subdivided into three categories depending on the level of toxicity, defined as low level: including minor

side effects such as increased cough, low fever (less than 37.5 °C, skin allergic reactions), medium level: including non-life threatening allergic reactions, infections that do not require hospitalization, elevation of liver enzymes or serum creatinine and high level: including death and/or life threatening major adverse events such as acute exacerbations. Thus primary outcome measures include safety with monitoring of serious adverse events.

2.7. Exploratory secondary end-points

Assessment of pulmonary function tests like Forced Vital Capacity (FVC %) predicted, diffuse lung capacity for carbon monoxide (DLco %) predicted, exercise capability 6 MWD as well as health-related quality of life questionnaires (SGRQ) at baseline. The patients were treated with a single dose of $\sim 13 \times 10^6$ cells, monitored throughout the infusion and then followed through 1 m, 4 m, 9 months for changes from baseline in lung function (FVC% and DLco%), Spo_2 and 6MWD.

2.8. Statistical analysis

Statistical analysis was performed using the SPSS 16.0 software. Safety and exploratory efficacy secondary endpoints were observed for each patient against the baseline values. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Patient recruitment status

Male and female IPF subjects were screened for the study and enrolled to receive BMSC's infusion following the eligibility criteria and were under follow-up. The following results depict the 9-months safety data in comparison to the baseline.

3.2. Patients' demographic data

All enrolled patients (n = 6), completed central line infusion. In addition, there was no patient that was terminated early in the study period. The majority of patients were males (n = 4, 1.5%) and the mean age was found to be 59 ± 07 yr and all patients were non-smokers. Most of the cases had hypertension (n = 2; 33%) and all patients were characterized with characteristic cough while one patient (2%) even had asthma and COPD (chronic obstructive lung disease). In these cases, diagnosis of IPF was based on both radiological and histological criteria. IPF diagnosis in this group of patients was set before current guidelines were published. All patients were of mild to moderate disease severity based on functional data with baseline values in FVC %
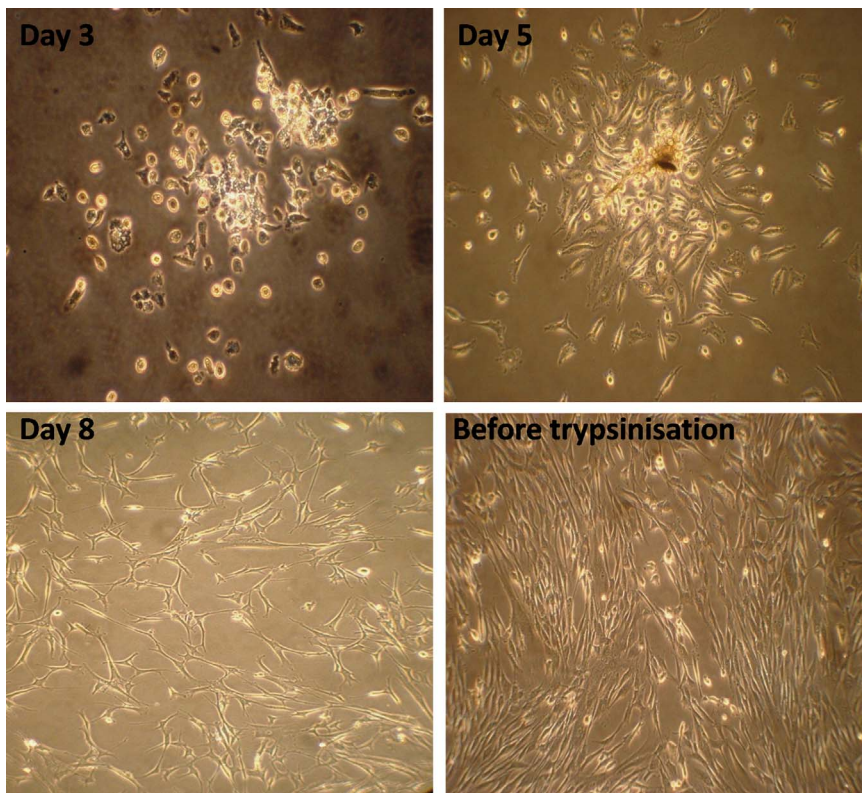
Fig. 1. Morphology of mesenchymal stem cells before and after trypsinization. MSCs demonstrated spindle shape by day 8 and were passaged on reaching 70% confluence under inverted phase contrast microscope.

predicted 33.9 ± 3.6 and DL_{CO} % predicted 27.1 ± 7.5.

3.3. Morphology of BMSCs

The BMSCs appeared ovular in shape till day 3 as shown in the Fig. 1. Cells attained spindle shape on 70% confluence and were trypsinized for passaging. Cells with normal and uniform distribution presented with visible fluorescence when observed under an inverted microscope.

3.4. BM derived MSCs differential cell count and viability

Isolated cells were analyzed for their viability and expression of stem-cell- specific surface antigens. The MSCs were found to be 98% viable with 7AAD. The yield and viability of the MSCs are represented in Table 2.

In line with previous reports, [12,13] flow cytometry analysis in our study revealed that the majority of isolated cells were positive for mesenchymal markers including, CD73: 87.5% and CD90: 89.6% while they were negative for CD45: 19.2%, CD34: 20.3% and HLA DR: 10% indicating the absence of hematopoietic and endothelial precursors (Fig. 2a).

Table 2
Viability and yield of the MSCs for infusion.

S.No	Age/Sex	Bone Marrow Aspirated(mL)	% Viability	MSC yield for infusion	Passage #
1	64/F	45	94	$14*10^6$ cells/mL	P_2
2	54/M	50	98	$12*10^6$ cells/mL	P_2
3	62/M	50	98	$12*10^6$ cells/mL	P_2
4	67/M	50	98	$12*10^6$ cells/mL	P_2
5	45/M	40	98	$12*10^6$ cells/mL	P_2
6	64/F	40	98	$13*10^6$ cells/mL	P_2

3.5. Tri lineage differentiation

The tri-lineage potential of BMSCs across the endoderm, ectoderm, and mesoderm showed differentiation to adipocytes, osteocytes, and chondrocytes respectively upon 21 days. The cell phenotype was confirmed using oil red o, von Kossa, and alcian blue for adipocytes, calcium deposition with nodule formation (osteocytes), and chondrocytes, respectively, under optical microscopy (Fig. 2b). The sterility check was performed as in-process quality control procedure by Hoechst staining using confocal laser scanning microscopy (CLSM). The sterility check for the medium used in the cultures was negative for bacterial and fungal contamination including mycoplasma (Fig. 2c).

3.6. Safety outcomes

An acceptable safety profile of stem cell infusions was reported in the enrolled patients. All the patients endured the infusions without any serious or clinically significant side effects during the entire study period. They had no allergic reactions, liver or renal abnormalities, and oxygen de-saturations. Cardiac abnormalities such as electrocardiogram or heart rate changes were observed to be in the normal limits. None of the patients experienced any ectopic tissue formation as reported by whole body CT scan that was performed at the end of follow-up period, meaning 9 months after the stem cell administration.

3.7. Efficacy outcomes

Decreased forced vital capacity (FVC%) was observed at baseline (33.9 ± 3.6) which increased during follow-up (1st month 45.8 ± 13.7, p = 0.02; 6th month 45.3 ± 8.1, p = 0.001; 9th month 45.7 ± 14.2, p = 0.03) (Fig. 3a). Gas exchange impairment also appeared as a restrictive impairment and diffusing capacity of carbon monoxide (DLco %) decreased. There was no statistically significant difference in one of the studied functional parameters (DL_{CO}% predicted; 27.1 ± 7.6) at baseline as compared to 1st month (32.2 ± 10.8) and 6th month
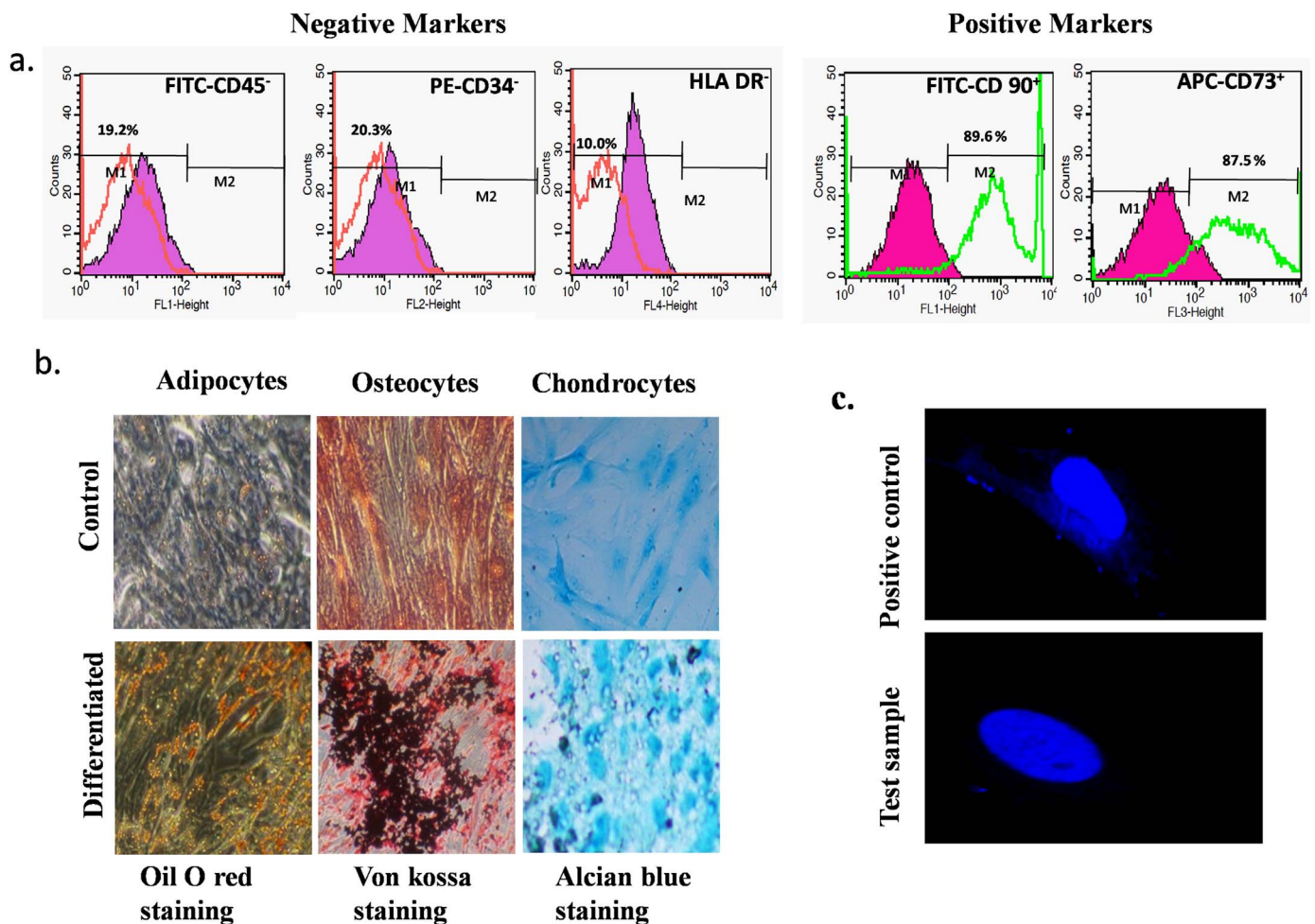
Fig. 2. a. Flow-cytometry analysis for the positive markers and Negative markers. Positive markers (CD90+, CD73+) and negative markers (CD34-, CD45- and HLA DR-) were indicated by peak shift towards the right side (M2), and left side with respect to unstained cells (M1) respectively. **b. Tri-lineage potential of BMSCs and BMSC surface marker characterization as quality check.** BMSCs demonstrated tri-lineage differentiation potential i.e., adipogenic, osteogenic, and chondrogenic and confirmed using oil O red, von kossa, and alcian blue staining respectively. **c. Sterility testing by Hoechst staining using CLSM.** The positive control shows mycoplasma contamination around the stained cells as dots whereas the test (patient) sample shows no contamination, indicating the sterility of the culture.

(33.2 ± 8.9) following infusion of BMSCs, however, statistically significant increase was observed at 9th month (36.4 ± 9.1; p = 0.03) (Fig. 3b). In addition, no significant alterations in dyspnea scale were noted at both serial time points of treatment follow-up period, as well as prior treatment initiation. Furthermore, significant difference was observed in 6MWD at 1, 6 and 9 months post- infusion (mean improvement of 84 m, 287 m and 292 m respectively) (Fig. 3c).
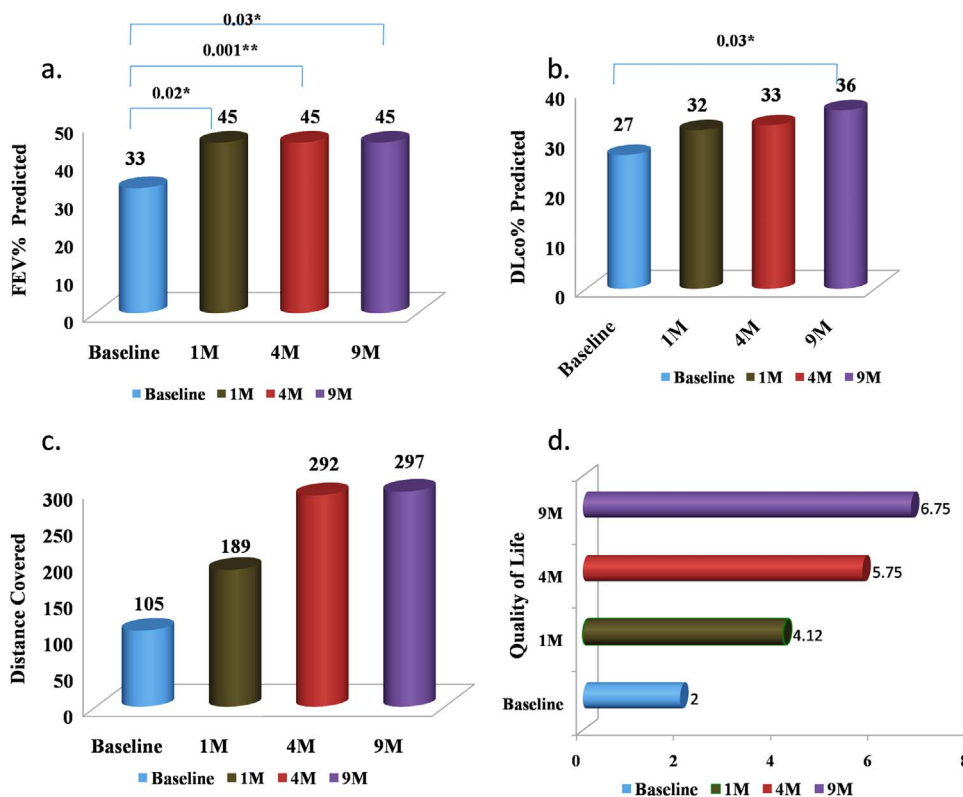
3.8. Quality of life

On following the quessionaire regarding the quality of life (QoL) values based on Saint George's Research Quessionaire-SGRQ, patients were found to exhibit extremely poor QoL before therapy and substantially improved following recovery. The progress in QoL indicated their recovery in the course of time, highlighting the importance of MSCs. After BMSC treatment, improved quality of life experienced by the patients should be seen in addition to any life extension achieved (Fig. 3d).

4. Discussion

The only intervention that improves survival in selected patients with IPF earlier is lung transplantation. In a study of 46 patients awaiting lung transplantation, survival increased by 79% one year post transplant and the relative risk reduction for those who underwent lung transplantation was 75% compared to patients who remained on the waiting list [14]. Despite its success, lung transplantation is not without significant risks. The most common complications and causes for poor long-term survival after transplantation include infection, acute and chronic graft rejection and airway stenosis [15]. Data from the International Society for Heart and Lung Transplantation (ISHLT) demonstrated that 1-, 2- and 3- year survival rates after lung transplantation in IPF were 80%, 66.7% and 53.3% respectively [16]. With lung transplantation currently being the only stable treatment option with a limited success rate, IPF patients and caregivers are hoping for the success of stem cell clinical trials in order to ensure a positive regenerative therapy. It was observed that the central line infusion of autologous BMSCs showed an acceptable safety profile in patients with IPF. This should be corroborated with larger cohort studies [17,18]. The other safety parameters of LFT, serum creatinine, and serum electrolytes were observed to be within the normal range [19–21].

With respect to safety concerns, possible risks that can be envisaged are pulmonary embolism, cardiac arrhythmias, and worsening of disease due to potential pro-fibrotic effects of MSCs [22,23]. Typical multipotent MSCs are characterized by their immunomodulatory, tissue protective properties, and their ability to manipulate the local milieu of damaged tissue in the lungs thereby reducing inflammation and promoting tissue repair, supporting the current focus for their use in IPF

Fig. 3. **Graphical representation in lung function changes and exercise ability in IPF patients.** The bar graph illustrates changes in a) FEV%, predicted b) DLco % of predicted and c) 6 MWD for baseline, 3-month, and 9-month follow-up. d) Quality of life scale denotes health perspective i.e., 1 as worst and 10 as excellent. DLco%, FEV% and QoL were performed at baseline, 4-month, and 9-month follow-up visits as per protocol.

disease studies [24,25]. The immunomodulatory properties of the other mechanisms proposed by researchers across the globe in protecting against acute lung injury are through mitochondrial transfer and cell fusion. In mitochondrial transfer, the infused MSCs increase alveolar ATP concentration by forming gap junction channels with alveolar epithelia and releasing the mitochondria containing micro vesicles, thus restoring bio-energetics of the injured alveolar tissue [26,27]. In IPF, the resident epithelial stem cells lose their renewal potential due to shortening of the telomere in response to negative environmental factors (such as smoking, pollution, and excessive use of corticosteroids). This results in the loss of epithelial coherence and unusual alveolar re-epithelialization [28]. When adult BMSCs are infused in fibrotic lungs, they have protective effects mediated by the release of paracrine factors. Deposition of the extracellular matrix (ECM) is prevented by these secreted factors, inhibiting the ECM-releasing myofibroblasts [29]. The infused BMSCs mediate the release of factors that reduce infiltration of inflammatory cells and the production of fibrotic mediators, thus re-generating lung epithelial and endothelial cells mitigating damage and fibrosis. Although the exact mechanism of action of MSCs in fibrosis is not clearly understood, immune modulation, inhibition of the epithelial to mesenchymal transition of alveolar cells to ECM-secreting myofibroblasts mediated by TGF-β, matrix remodeling, and oxidative stress inhibition are proposed mechanisms of action of BMSC's [29].

We examined an increase in the FVC % unlike the annual decline in another study in seven patients with idiopathic pulmonary fibrosis and found that the yearly decline of FVC% was 20.3% [30], which seems larger than that observed in IPF, although the number of patients included in the study was small. Rapid decline of FVC% is related to the prognosis of pulmonary fibrosis [31], while the degree of annual decline may depend on the stage of disease at the start of examination of the annual change. Further studies are warranted to support the observation. We have seen an elevation in the DLco% unlike the study conducted in USA [32]. In addition to 9 months follow-up, data

revealed a marginal improvement at 6MWD and FVC% levels, similar to a Greece population study [33,34]. Administration of MSCs would eventually increase respiratory and forced volume capacity and contribute to improved lung health.

MSC treatment for fibrotic pulmonary disease appears to be efficacious. In fact, one of the earliest studies documenting therapeutic efficacy of MSC infusion was in mouse models of bleomycin-induced lung fibrosis, which is an animal model for IPF [35]. Subsequently, the same group demonstrated that MSC secreted IL-1 receptor antagonist mediated the anti-inflammatory and anti-fibrotic effects [36]. Using the same disease model, infusion of umbilical cord MSCs were also shown to have therapeutic effects [37]. In addition to anti-inflammatory effects, MSC treatment may reduce fibrosis through enhancing the resident lung bronchio-alveolar stem cell population for repair and regeneration of healthy lung parenchyma [38]. Such profound effects induced by MSC treatment may account for the rapid push to clinical studies in this field, since about half of the basic and animal studies in this field were published within the past 3 years. Our study seems promising and intriguing where none of the patients enrolled experienced any major side effects.

5. Conclusion

Our study was conducted to assess the safety of central line infusion of BMSCs in IPF patients wherein we met our primary objective and indicated an acceptable safety profile. Detailed safety monitoring through several time-points indicated that cell-treated patients did not deteriorate, as assessed by functional parameters and indicators along with quality of life, wherein we met our secondary objective of efficacy. Our findings provide a way forward in carefully designed, efficacy trials investigating the therapeutic use of cell-based therapies in patients with end stage lung diseases, including IPF overcoming steep barriers such as ethical issues and safety concerns.

In conclusion, the study has the potential in furthering the clinical trials to Phase I & II in a larger cohort. The pilot data suggests that the single dose infusion of 13×10^6 cells/mL, through central line route of administration can improve the QoL and may increase the therapeutic window period for lung transplant.

Executive summary

- Mesenchymal stromal cells demonstrated that they can provide intermediate improvement in QoL and alleviate the morbid conditions of the idiopathic pulmonary fibrosis patients. They contribute to the improved lung health by increasing the respiratory health.
- Central line is considered as a viable route of infusion owing to its safe bed-side practice and optimizes the maximum cell delivery obviating migration to other organs and compromised cell viability.
- Autologous BMSC therapy is devoid of expensive immune suppressive drugs.
- Study warrants a larger cohort to corroborate the findings.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical clearance

All the ethics and institutional regulatory approvals (IEC-institutional ethics committee and IC-SCR- institutional committee for stem cell research) were obtained prior to start of the trial. The trial is also registered in public trials registry (www.ctri.nic.in CTRI/2015/02/005569).

Author contributions

Upasna Upadhyay, Chandrashekar Mallarpu, Meenakshi Ponnana: Manuscript writing, performing the experiments, management and assembly of clinical data;

Ravindra Nallagonda, Sudhir Prasad, Mohammed Samiuddin, Rajat Mohanty: Patient screening, recruitment, patient management, data analysis and interpretation;

Sindhoora Rawul: Pulmonary function assessment;

Eswara Prasad Chelluri: Supervised PFT assessment and reviewed the manuscript;

Lakshmi Kiran Chelluri: Concept and design, manuscript writing and final approval of manuscript.

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