CLINICAL TRACK

Endothelial NO-Synthase Gene-Enhanced Progenitor Cell Therapy for Pulmonary Arterial Hypertension: the PHACeT Trial

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ABSTRACT

Rationale: Pulmonary arterial hypertension (PAH) remains a progressive and eventually lethal disease characterized by increased pulmonary vascular resistance due to loss of functional lung microvasculature, primarily at the distal (intracinar) arteriolar level. Cell-based therapies offer the potential to repair and regenerate the lung microcirculation and have shown promise in pre-clinical evaluation in experimental models of PAH.

Objective: The Pulmonary Hypertension And Cell-Therapy (PHACeT) trial was a phase 1, dose-escalating clinical study of the tolerability of culture-derived endothelial progenitor cells (EPCs), transiently transfected with endothelial NO-synthase (eNOS), in patients with PAH refractory to PAH-specific therapies.

Methods and Results: Seven to 50 million eNOS-transfected EPCs, divided into 3 doses on consecutive days, were delivered into the right atrium via a multiport pulmonary artery catheter during continuous hemodynamic monitoring in an ICU setting. Seven patients (5 female) received treatment from December 2006 to March 2010. Cell infusion was well tolerated, with no evidence of short-term hemodynamic deterioration; rather, there was a trend towards improvement in total pulmonary resistance (TPR) over the three-day delivery period. However, there was one serious adverse event (death) which occurred immediately after discharge in a patient with severe, end stage disease. Although there were no sustained hemodynamic improvements at 3 months, 6MWD was significantly increased at 1, 3 and 6 months.

Conclusion: Delivery of EPCs overexpressing eNOS was tolerated hemodynamically in patients with PAH. Furthermore, there was evidence of short-term hemodynamic improvement, associated with long-term benefits in functional and QOL assessments. However, future studies are needed to further establish the efficacy of this therapy.

ClinicalTrials.gov Identifier: NCT00469027

Keywords: Pulmonary Hypertension; cell therapy; gene therapy; endothelial progenitor cells
Nonstandard Abbreviations and Acronyms:

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a devastating disease that is caused by a progressive increase in pulmonary vascular resistance (PVR), largely due to severe remodeling of distal lung arterioles. Recent evidence from experimental models has strongly implicated endothelial cell (EC) injury and apoptosis as a critical trigger for this disease. Loss of ECs at the level of the fragile precapillary arteriole could result in arteriolar dropout and disruption of the arteriolar-capillary circulation. As well, reactive proliferation of remaining vascular cells may result in arteriolar obliteration and the formation of complex plexiform lesions, further contributing to the loss of effective lung microvascular area. Current PAH-specific therapies have only limited ability to reverse lung arterial remodeling and have not been curative. Thus, new approaches that have the potential to restore the damaged lung microcirculation need to be explored.

Endothelial progenitor cells (EPCs) are released from the bone marrow and home to regions of vascular injury or ischemia to participate directly in revascularization and tissue repair, or release paracrine factors which stimulate the local vascular repair and regeneration. The importance of EPCs in systemic revascularization has been demonstrated in experimental models of hindlimb ischemia and myocardial infarction. More recently, a number of clinical trials have been performed to study the safety and efficacy of progenitor cell therapy, with promising results for the treatment of acute myocardial infarction and hindlimb ischemia.

However, less is known about the role of EPCs in the pulmonary vasculature. Our group and others, have shown that the administration of culture-modified blood-derived mononuclear cells commonly referred to as early out-growth EPCs (or circulating angiogenic cells, CACs) could prevent the increase in pulmonary arterial pressures as well as arterial and right ventricular remodeling in experimental models of PAH; although their therapeutic efficacy was more modest in treatment models in which cell therapy was delivered in the context of established PAH. We have further demonstrated that transfection with endothelial NO-synthase (eNOS) significantly enhanced the ability of EPC to reverse hemodynamic and remodeling abnormalities in the monocrotaline (MCT) treatment model of established PAH. Even somatic cells, such as fibroblasts, were effective in improving pulmonary hemodynamics and microvascular perfusion in the MCT treatment model when transfected with eNOS, consistent with the well-recognized role of NO in vascular repair and regeneration. Moreover, eNOS expression and activity has previously been shown to correlate with the regenerative activity of progenitor cells in the systemic vasculature.

The Pulmonary Hypertension and Angiogenic Cell Therapy Trial (PHACeT) was a “first-in-human”, phase 1 dose-escalation study examining the tolerability and potential efficacy of eNOS gene-enhanced progenitor cell therapy for PAH. We now report that administration of eNOS-transfected early outgrowth EPCs into the pulmonary circulation of patients with stable severe PAH was well tolerated, and may have resulted in short-term hemodynamic improvements as well as sustained increases in exercise capacity.

METHODS

The PHACeT trial was an open label, dose-escalating protocol. Patients were enrolled in two Canadian sites. In Toronto, patients were referred from the Pulmonary Hypertension Program of the University Health Network (UHN) and underwent study treatment and follow up at St. Michael’s Hospital (SMH). In Montreal, patients were identified and enrolled at the Center for Pulmonary Vascular Disease, Division of Cardiology, Jewish General Hospital (JGH), McGill University. The study was approved by local Research Ethics Committees at each institution and overseen by an independent, blinded DSMB.
patients provided written informed consent. The inclusion criteria required that patients had: WHO group 1 Pulmonary Arterial Hypertension (PAH); specifically, idiopathic, associated with systemic sclerosis, anorexigen exposure, or repaired atrial septal defect; with WHO functional class III or IV symptoms despite treatment with conventional therapies including intravenous epoprostenol at maximal tolerated doses; and 6-minute walk distance (6MWD) between 150 meters and 400 meters. A complete list of inclusion and exclusion criteria is provided in Online Table I.

**Manufacture of eNOS-transfected early outgrowth EPCs**

Cell processing was performed at the Orsino Cell Processing Facility at the UHN in Toronto and at the Lady Davis Institute Facility at JGH in Montreal as described in the online supplement.

**Delivery of eNOS gene-enhanced EPCs**

All patients were admitted to hospital electively for insertion of a multiport pulmonary arterial catheter via a central vein. The cell suspensions were injected by hand at a rate of no more than 2 ml/minute at a concentration of 2.5 million cells/ml. Full hemodynamics, including CO and PAWP, were recorded prior to and 30 minutes post each cell delivery as described in the online supplement and pulmonary arterial pressures were continuously monitored during cell delivery. For further safety, the total cell dose for each panel was divided into 3 separate aliquots delivered on consecutive days as detailed in Figure 1. After the first cell injection, patients were transferred to a critical care unit and cell administration was repeated on the day 2 and 3 with full hemodynamic assessments before and after each cell delivery. A total cell dose of 7, 23 and 50 million cells was delivered for panels 1, 2 and 3, respectively.

**Biomarkers and cytokines**

Blood samples for measurement of N-terminal pro-brain natriuretic peptide (NT-proBNP), C-reactive protein (CRP) and interleukin-6 (IL-6) were obtained at baseline, after each injection during the cell delivery period, predischarge (day 4) and at each follow up visit. NT-proBNP and CRP were measured by the clinical laboratory for each site, whereas IL-6 was assessed by ELISA at a central facility. IL-6 was measured using the Biosource human IL-6 US, UltraSensitive assay #KHC0064 (BioSource International, Camarillo, CA, USA) or the Quantikine HS ELISA for Human IL-6, #HS600B (R&D Systems, Inc, Minneapolis, MN, USA).

**Statistical analysis**

For this small, phase I study, we used descriptive statistics (means and standard deviation) to assess safety and tolerability. To evaluate preliminary evidence of effectiveness, we used repeated measures ANOVA as well as general linear regression analyses of outcomes measured repeatedly over time, after making normalizing transformations where necessary and visually verifying that normality assumptions were reasonable. In the general linear regression analyses, time was modeled using a fixed categorical covariate, and regression parameters were estimated using Restricted Maximum Likelihood Estimation with degrees of freedom estimated using the Kenward-Roger method as recommended for small samples. The variance-covariance matrix was modeled to account for correlation in repeated measures on the same patient over time. Analyses were conducted using all available data on each patient. To examine the potential impact of missing data, sensitivity analyses were conducted using single imputation under conservative assumptions about the missing responses. Analyses were conducted using the Mixed procedure in SAS v.9.2 as well as GraphPad Prism (version 5 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com).

**Role of the funding source**

The study was sponsored by Northern Therapeutics Inc. DJS acted on behalf of the sponsor to ensure that the study was conducted in compliance with all regulatory and GCP requirements, but otherwise refrained from any direct involvement with patient enrolment or care. All data were analyzed by an independent statistical consultant (MT) at the Ottawa Methods Centre of the Ottawa Hospital Research Institute.
RESULTS

Patient characteristics:
A total of seven patients with idiopathic PAH were enrolled between November 2006 and March 2010. Five female and two male patients were included in the study, with a mean age of 52±20 years (Table 1a). All patients had at least WHO class III symptoms, and their mean 6MWD was 361±110 meters. All but the first patient were on dual oral PAH therapies (endothelin receptor antagonist and phosphodiesterase 5 (PDE5) inhibitor), or intravenous epoprostenol with or without sildenafil. Table 1b summarizes the hemodynamic data obtained just prior to cell delivery. One patient (01-001) did not meet hemodynamic criteria at the time of the baseline hemodynamic assessment; however, eligibility as defined by the protocol was based on diagnostic right heart catheterization performed up to a year prior to enrolment. Mean pulmonary arterial pressure (mPAP) at baseline was 55±13 mm Hg (median 57 mm Hg), with a cardiac output (CO) of 4.91±1.87 L/m (median 5.47 L/m) and a calculated total pulmonary resistance (TPR) of 1062±585 dynes/sec*cm^5 (median 800 dynes/sec*cm^5) (Table 1b).

Short-term effects on pulmonary hemodynamics and gas exchange:
Hemodynamic parameters were monitored before and after each cell infusion (Figure 2A and B). In the majority of patients, mPAP and TPR declined over the 3-day cell delivery period, while CO rose. The only exception was the patient with the highest CO and the lowest PVR and TPR at baseline (Patient 7, Table 1b; Figure 2; double arrow). Overall, the decrease in TPR over the cell delivery period showed a statistical trend for all 7 patients (p=0.06). No cell dose-effect relationship was seen (Online Figure I); in fact, the effect seemed to be greatest in the first dose panel and the single patient entered into panel 3 was the only one to show an increase in TPR over 3-day delivery period. However, in absolute terms the increase was very modest, and thus the percent change was exaggerated since this patient had the lowest baseline TPR. Interestingly, the three patients receiving background sildenafil therapy, alone or in combination therapy, were among those with the greatest improvements in hemodynamics (Figure 2 A-C, open symbols), reaching significance for TPR (p<0.05; two-way RM ANOVA). Importantly, there were no significant changes in gas exchange as assessed by arterial O2 partial pressure and saturation over the 3-day treatment period (Online Table II).

Adverse events:
Two serious adverse events (SAEs) occurred during the one-year follow up period, including a death (patient 5) soon after discharge. The patient collapsed suddenly on arrival home, and could not be resuscitated by emergency personnel. This patient had a history of recurrent pre-syncope and frequent admissions for right heart failure, in addition to other features consistent with poor prognosis (Table 1). He was the second patient to receive a total of 23 million cells and showed no acute hemodynamic deterioration during or immediately following cell product delivery (Figure 2; single arrow). There were no obvious changes in vital signs or oxygen saturation during the course of the admission and no evidence of pulmonary arterial rupture or emboli was found on autopsy, which disclosed some interstitial fibrosis and patchy honey-comb lung. However, the pulmonary function test was normal, apart from marked reduction in diffusing capacity, and the chest CT scan showed only mild interstitial fibrosis. This event was deemed by the DSMB to be “possibly related” to cell therapy, which was the lowest level of causality as per predefined criteria in the protocol. The second SAE was for sepsis leading to hospitalization (patient 3), which occurred 9 months after cell delivery and was deemed unrelated to the cell product. After completion of the 1-year follow up period, there were two more deaths, at 2.6 years (patient 2) and 4.6 years (patient 3) post cell delivery, as well as one patient who developed breast cancer 4 years post cell therapy. In addition, there were six hospitalizations occurring between 23 to 32 months post cell delivery involving three individual patients. Four of these were for right heart failure occurring in the same patient (patient 2) in the months leading up to her death, and precipitated by withdrawal of one of her PAH-specific medications. The other two hospitalizations were for atrial fibrillation and a febrile condition, respectively, both occurring more than 2 years post treatment. Other adverse events (AEs) that
were reported during study period are summarized in Online Table III. There was an average of 15±7 AEs per patient (range 5 to 24). The most frequently reported events were related the musculoskeletal, gastrointestinal, and respiratory systems, as well as access site pain/hematoma; although these were for the most part minor.

**Long-term functional, QOL and hemodynamic changes:**
Mean 6MWD improved significantly over the follow up period (Figure 3A; p=0.006), with a mean increase of 65 meters at 1 month (p<0.001), persisting at 3 and 6 months (48 and 47 meters, respectively; p<0.01). After imputing no change from baseline for the patient who died, the conclusions remained unchanged (p=0.007), with a mean increase of 54 meters at 1 month (p=0.001), persisting at 3 months (39 meters, p=0.012) and 6 months (38 meters, p=0.014). The statistically significant improvement in 6MWD persisted even after imputing a worsening of up to 20% for the patient who died (p=0.032). Two patients demonstrated improvement in WHO functional class (FC) from class III to class II at 3 and 6 months of follow up (Figure 3C) and this increased to three patients at the last follow up assessment (27±16 months post treatment). In addition, there was a highly significant increase in the physical component summary (PCS) measure of the SF36 QOL score (Figure 4; p<0.0001), whereas the mental component summary (MCS) was not changed. Individual component scores are shown in Online Figure II. However, there were no significant differences in the hemodynamic parameters between baseline and 3 month follow up in the six patients that had repeated catheterizations (Figure 5). As well, there was no significant change in pulmonary function over a 6-month follow up (Online Table IV).

**Effects of cell therapy on cytokines and biomarkers:**
No significant changes were apparent in NT-proBNP levels during the 3 day cell delivery period (Figure 6A), or during the 6 month follow up period; despite a tripling in levels on Day 4 for one patient (Patient 5; indicated by the arrow). There were significant, albeit modest, increases in both CRP (Figure 6B, p<0.0001) and IL-6 (Figure 6C, p<0.05) during the cell delivery period, both of which peaked at day 4 (1 day post final injection of cell product), and returned to baseline by the week 1 visit and remaining at baseline levels for the 6 month follow up. Again, patient 5 showed the highest levels of CRP and IL-6 at baseline and over the cell delivery period.

**DISCUSSION**

The PHACeT trial establishes the feasibility and tolerability of eNOS gene-enhanced cell therapy for PAH. If anything, infusion of the eNOS-transfected EPCs was associated with short-term hemodynamic improvement, particularly in patients receiving a PDE5 inhibitor as part of their background therapy, and also sustained benefits in exercise capacity, QOL scores and symptom class.

While current pharmacological therapies for PAH provide benefit in terms of exercise tolerance and modest improvements in pulmonary hemodynamics 33-35, they do not appear to reverse the severe arteriolar remodeling which is characteristic of this disease 36 and, with the exception of epoprostenol, their effect on survival has not been well established 37, 38. Certainly, there is as yet no cure for PAH; novel therapeutic strategies are required to address the underlying structural and functional abnormalities driving the relentless progression of this disease 39.

Cell therapy for other cardiovascular diseases has been studied in a number of clinical trials and systematic reviews of this literature suggest a highly significant, albeit modest, benefit in terms of increase in left ventricular ejection fraction post MI 16, 40, as well as long-term improvements in clinical outcomes, including event free survival and mortality. Most cardiovascular cell therapy trials have used heterogeneous populations of bone marrow mononuclear cells (MNCs) 16, 17, 41, which are believed to
harbor a rare population of stem or progenitor cells \(^42\). Some studies have used progenitor cells selected on the basis of surface markers such as CD34 or CD133 \(^{43, 44}\), although there is no consensus regarding the definition of a true EPC population \(^45\). An alternative strategy is to derive angiogenic cells using defined culture conditions. When plated on fibronectin in the presence of endothelial growth factors, a subset of MNCs will attach and acquire a rod-like morphology by about three days of culture. These cells are termed early outgrowth EPCs \(^46\) or circulating angiogenic cells (CACs) \(^47\), and possess potent angiogenic properties.

The PHACeT trial is the first to assess the tolerability and potential benefits of eNOS gene-enhanced EPCs in patients with PAH. A previous randomized but nonblinded study using nonmodified early outgrowth EPCs showed modest hemodynamic and functional benefits at 3 months post treatment \(^48\). In that trial, EPCs were obtained from a simple peripheral blood draw yielding, on average, a total cell dose of ~11 million EPCs after 7 days of culture (4 to 23 million). In the PHACeT trial we harvested circulating mononuclear cells by apheresis without mobilization, and EPCs were transiently transfected with human eNOS plasmid DNA after ~7 days of culture to further enhance their activity.

Cell delivery did not adversely affect hemodynamics up to a total dose of 50 million cells. But all but one patient showed either no change, or even an improvement, in hemodynamics over the cell delivery period. However, by relying on a previous catheterization study to determine eligibility, one patient did not meet hemodynamic criteria for PAH at the baseline study, which may have reduced the ability to demonstrate improvement after cell therapy. In future studies, it may be advisable to use the baseline study for this purpose, even if this results in potential wastage of the cell therapy product. Nonetheless, there was a trend towards improvement in pulmonary vascular resistance, which could be attributed mainly to patients receiving concomitant background therapy with PDE5 inhibitors. Since inhibition of PDE5 will increase cGMP, thereby enhancing the biological effects of NO, this observation is consistent with an amplified effect of increased local NO production within the distal arteriole, possibly as a result of delivery of eNOS-transfected EPCs. While eNOS is better known for its role in vascular homeostasis and endothelial function \(^49\), its direct enzymatic product, NO, plays an important role in angiogenesis and neovascularization as well \(^50, 51\), acting as a key downstream mediator of the angiogenic effects of VEGF and other angiogenic growth factors \(^52\), as well as inducing the expression of VEGF \(^53\). Moreover, in a direct comparison with VEGF, somatic cell-based gene therapy with eNOS was more effective at reversing established PH in the MCT rat model \(^54\).

We also measured inflammatory cytokines throughout the study. Interestingly, there were transient increases in both CRP and IL-6 during and after cell therapy, which rapidly returned to baseline by the first week. However, the magnitude of these changes was modest compared to clinically significant inflammatory conditions, for example mean peak levels of IL-6 were at least of an order of magnitude lower than those reported in rheumatoid arthritis \(^55\) while peak CRP levels were within the range previously described in stable PAH patients \(^56\). Although it is unlikely that autologous cells themselves would induce an immune response, it is possible that residual plasmid DNA, which is known to contain bacterial DNA elements that can stimulate innate immune responses \(^57\), could be a contributing factor. It is also possible that to some extent these increases could be attributable to the invasive procedure and the insertion of an indwelling catheter \(^58\). To our knowledge, inflammatory cytokines have not been previously assessed after cell delivery in other cell therapy trials, despite the fact that many of these use allogeneic cell products \(^59\) or xenobiotic reagents (fetal bovine serum) \(^60\), which could be expected to induce an immune response.

While the acute cell delivery was hemodynamically well tolerated, one patient died suddenly soon after discharge raising potential safety concerns, particularly in a patient population with severe baseline hemodynamic compromise. Although this patient exhibited a number of features consistent with high risk and poor prognosis, there was no evidence of acute hemodynamic deterioration during or after cell
delivery in this patient (Figure 2). Nonetheless, we believe that the stress of invasive monitoring for several days in a patient in such a fragile hemodynamic state could have precipitated decompensation contributing to his sudden collapse. Indeed, this patient also exhibited basal cytokine levels that were amongst the highest of this population (Figure 6), and showed the greatest increases after cell delivery. Notably, this was the only patient that demonstrated a clear rise in NT-proBNP, as possible indicator of worsening right heart failure. In future studies of cell therapy it would be prudent to exclude patients with particularly high risk features (i.e., recurrent admissions for right heart failure, presyncopal episodes and excessive desaturation) and perform careful monitor of NT-proBNP levels before and after cell delivery to identify patients with subclinical hemodynamic deterioration.

Despite trends toward possible short-term hemodynamic improvements, there was no sustained improvement in pulmonary vascular resistance in this small study. However, the PHACeT trial was not powered for efficacy and a larger study with an appropriate randomized design would need to be performed to better assess the potential benefits of eNOS gene enhanced cell therapy in patients with severe PAH. As well, the transient transfection strategy used in this study only resulted in detectable eNOS transgene expression for less than one week. While transient transfection has the advantage of greater safety, future experimental studies are needed to explore whether stable transfection or multiple dosing can provide superior long-term improvement. Moreover, based on experimental studies, only a small fraction of the EPCs would be expected to remain within the lung beyond a few weeks. Thus, repeated cell delivery may be necessary in future trials for more robust long-term benefits. Nonetheless significant improvement in exercise tolerance was seen which was greatest at one month, but persisted for up to 6 months. This was associated with an improvement QOL scores over the course of follow up and a decrease functional class in half of the patients completing long-term follow up. Once again caution must be exercised when interpreting subjective data from such a small and noncontrolled open-label trial in which a placebo effect cannot be excluded.

This was a challenging trial to perform for many reasons, not the least on which was the difficulty in recruiting patients, and future cell or gene therapy studies will need to overcome such difficulties by including multiple study sites and enhancing physician and patient awareness. Despite its limitations, the PHACeT trial represents the world’s first study of gene-enhanced progenitor cells in the treatment of severe PAH. It demonstrated that cell-based gene therapy was hemodynamically tolerated, and may have resulted in a trend towards modest short-term improvements and sustained increases in function and symptom class. Future studies are warranted to better establish both safety and efficacy of eNOS gene-enhanced EPCs in PAH, and to explore the possibility that regenerative approaches may be able to restore pulmonary vascular structure and function in patients with advanced disease.
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DISCLOSURE
Dr. Duncan J. Stewart is the president of Northern Therapeutics, and has an equity interest in Northern Therapeutics. Dr. David Courtman is the Chief Scientific Officer of Northern Therapeutics.
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TABLE 1

a) Patient characteristics

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Mean 52

| STD | ±20 | ±110 | ±10 | ±219 |

b) Baseline hemodynamics

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Mean 8

| STD | ±5 | ±23 | ±8  | ±13 | ±1.9 | ±1.06 | ±6  | ±536 | ±215 |

* Patient was not eligible for parental prostaglandin therapy due to the remote location of his residence.

PAH specific medications: Bos - Bosentan, Sild - Sildenafil, Epo - Epoprostenol, Sita - Sitaxsentan; Other medications: War – Warfarin, Fur - Furosemide, Spir– Spironolactone, Dig - Digoxin
FIGURE LEGENDS

Figure 1: Panel 1 delivered a total number of 7 million cells divided into three separate injections of 1, 3 and 3 million cells, delivered on three sequential days. In panels 2 and 3, the total cell dose was 23 and 50 million cells, again delivered over 3 subsequent days.

Figure 2: Acute hemodynamic changes in patients receiving cell therapy. Measurements of mPAP (A), CO (B), and TPR (C) were made using PA catheter pre- and post-cell infusion, on the three consecutive days of cell delivery immediately before cell injection (circles) and 30 minutes post (squares). Absolute values for each patient are shown the left-sided panels, whereas relative (%) change is presented on the right. Open symbols denote patients that were receiving concomitant therapy with a phosphodiesterase type 5 inhibitor. Statistical analyses were performed using RM-ANOVA. No significant changes in mPAP or CO were noted; however, a trend towards improvement in TPR was observed over the 3-day course of cell infusion (p=0.06). → and => denotes patients 5 and 7, respectively.

Figure 3: Long term functional assessment of patients receiving gene-enhanced cell therapy. Change in 6MWD at 1, 3 and 6 months of follow up A) for each patient, and B) mean data and SEM. Statistical analyses were performed using RM-ANOVA, with post analysis using the Man-Whitney test. C) WHO functional class at 1, 3 and 6 months of follow up, and at the final visit (27±16 months post treatment). ** = p<0.01, * = p<0.05.

Figure 4: Long term SF-36 quality of life assessment. A) Physical Component Summary (PCS) and B) Mental Component Summary (MCS), at baseline and over a 6 follow up period. Statistical analyses were performed using general linear regression analyses.

Figure 5: Long-term hemodynamic assessments. A) CO, B) mPAP, and C) TPR measured immediately before cell delivery (Baseline) and 3 months of follow up. No significant significances were seen in the 6 patients that completed follow up.

Figure 6: Changes in biomarkers and cytokine levels. A) N-terminal brain natriuretic peptide (NT-BNP), B) C-reactive protein (CRP) and C) interleukin-6 (IL-6) measured immediately before cell delivery (Baseline) and over the course of cell therapy and subsequent follow up. ← denotes patient 5. Changes in CRP and IL-6 over the first 4 days were significant (p<0.0001 and p<0.005, respectively.)
Novelty and Significance

What is known?

- Pulmonary arterial hypertension (PAH) results from the extensive loss of small blood vessels in the lung.
- Current medical treatments only modestly improve lung circulation.
- Endothelial progenitor cells (EPCs) are believed to circulate in the blood and act to repair and regenerate damaged blood vessels.
- It is not known whether EPCs can restore functional lung circulation in patients with PAH.

What new information does this article contribute?

- This is the first clinical study using EPCs genetically engineered to increase their production of nitric oxide, an endothelial vasodilator factor necessary for blood vessel growth, in patients with PAH.

Despite recent advances in medical treatment, there is no cure for PAH and the long-term outlook remains poor. Patients with PAH exhibit a marked reduction in the number of small blood vessels in the lung; therefore, we hypothesized that a regenerative approach to restore lung vasculature might represent a promising new therapy for this disease. The PHACeT trial is the first clinical pulmonary hypertension study to use genetically enhanced EPCs, which are designed to repair and regenerate blood vessels. This study shows that gene-enhanced EPCs can be given to the lung circulation of patients with PAH without immediate adverse hemodynamic effects. One patient with severe pulmonary hypertension died shortly after hospital discharge. Patients with very advanced disease and indications of high risk may not be suitable for this therapy. In most patients, there was a modest short-term improvement in pulmonary arterial pressures. There were significant and persistent benefits in the mean walking distance and quality of life scores for up to 6 months. This study suggests feasibility of administering genetically modified eNOS to patients with severe PAH without immediate adverse hemodynamic effects. Future clinical trials are needed to support utility of this innovative approach in restoring functional lung blood vessels, as a potential transformative therapy, in patients with PAH.
Figure 1

Escalating cell dosing panels

<table>
<thead>
<tr>
<th>Panel</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<tr>
<td>Panel 3</td>
<td>10x10^6</td>
<td>20x10^6</td>
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<td>Panel 2</td>
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<td>Panel 1</td>
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<td>3x10^6</td>
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</table>

Day 1 Day 2 Day 3

3 patients/panel, 6 patients in the final panel
Figure 2
Figure 4
Figure 5
Figure 6
Endothelial NO-Synthase Gene-Enhanced Progenitor Cell Therapy for Pulmonary Arterial Hypertension: the PHACeT Trial
John Granton, David Langleben, Michael J Kutryk, Nancy Camack, Jacques Galipeau, David Courtman and Duncan J Stewart

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Supplemental Material

Inclusion criteria:
Patients participating in the study must have PAH and meet all of the following inclusion criteria:

- Male or female ≥ 18 years, ≤ 80 years
- Clinical diagnosis of idiopathic PAH (IPAH), familial PAH (FPAH) or anorexigen induced PAH, confirmed on any prior heart catheterization
- Class 3 or 4 symptoms (WHO), despite treatment with conventional therapies at the maximal tolerated doses
- 6-minute walk distance ≥ 150 meters and ≤ 400 meters
- Female patients who are surgically sterile, or are postmenopausal, or have documented infertility, or are of childbearing potential using one of the following methods of contraception: Barrier-type devices (e.g. condom, diaphragm) used in combination with a spermicide. A double-barrier method is recommended; intrauterine devices (IUDs); oral or implanted contraceptives, if used in combination with a barrier method
- Provided written informed consent

Exclusion criteria:
Patients will not be included in the study if any of the following exclusion criteria are present:

- Intra or extra cardiac communication between the right and left sided circulations (including PFO) with demonstrated right to left shunting
- Hemodynamic instability (i.e. intravenous inotropes, ventilatory support, etc.)
- Left ventricular ejection fraction < 40%
- Documented thromboembolic event in last 3 months
- History of pulmonary embolism or chronic thromboembolic PAH (must have negative ventilation perfusion scan or CT angio)
- Hospitalization for worsening right sided heart failure within 3 months of screening
- Introduction of endothelin receptor antagonists (ERA), prostaglandins, phosphodiesterase inhibitors or calcium channel blockers within 3 months prior to screening. Minor adjustments of current medications will not disqualify patients.
- Pregnancy (by β-hCG or other testing) or nursing mothers
- History of cancer in the past 5 years (except for low grade and fully resolved non-melanoma skin cancer)
- Positive HIV test
- Concurrent Syphilis (positive VDRL)
- Concurrent hepatitis B or C (HBsAg, HB Core, HC Antibody)
- Concurrent West Nile Virus
- Autoimmune disorders (SLE, scleroderma, mixed connective tissue disease)
- Serum Creatinine>150 µmol/L
- Evidence of active infection (WBC >13,000, temperature >38.5C, or infiltrate on chest x-ray)
- AST and /or ALT values greater than 3 times the upper limit of normal
- Restrictive lung disease (TLC or FVC <60%) and/or obstructive lung disease (FEV1/FVC ratio <50%)
- CVP > 20 mm Hg at time of research heart catheterization
- Systolic blood pressure < 85 mm Hg
- Planned surgical intervention during the study period (excluding lung transplantation)
- Known allergy to gentamycin or amphotericin
- Patients receiving other investigational drug or device therapy within 30 days of screening
- Patients who have participated in any gene therapy study or an angiogenic growth factor protein study
- Patients unable to provide informed consent and comply with the visit schedule

Safety Variables and Analysis:
The primary endpoints will be related to the tolerability and safety of injection of genetically engineered endothelial progenitor cells in patients with severe PAH. These will be divided into early and late endpoints.

**Early (prior to hospital discharge):**
- Clinically significant changes in RAP, PAP, PVR, or SVR, or decrease in CO
- heNOS plasmid detection in systemic arterial blood (quantitative PCR) pre and post cell delivery
- Evidence of allergic reaction to cell injection defined as skin rash or wheezing
- Evidence of any systemic embolization during the hospitalization period

**Late (up to 6 months post cell delivery):**
- Pulmonary hemodynamics at 3 months (RAP, PAP, PVR, SVR and CO)
- Echo estimate of RVSP at 3 months and the other follow up visits
- Non-invasive assessment of pulmonary arterial-venous shunting by contrast echo
- PAH symptom class (WHO)
- Time to clinical worsening (death, hospitalization for right heart failure, etc.)
- Exercise tolerance (6 minute walk): before and at 1, 3 and 6 months
- Dyspnea as assessed by the Borg Dyspnea index
- Pulmonary function with DLCO
- Changes in ventilation perfusion scan
- QOL measures: SF36 at 1, 3 and 6 months
- Immune surveillance (BNP, IL-6, CRP, ESR, complement, CBC with differential: baseline, 1 week, 1, 3 and 6 months)
- Other: the dose of medications, need for transplant, ECG
Manufacture of eNOS-transfected early outgrowth EPCs
Mononuclear cells were isolated from circulating blood by apheresis (COBE Spectra™, Terumo, BCT, CO) and early outgrowth EPCs were selected by differential culture on human fibronectin coated flasks in the presence of recombinant human growth factors (Bullet kits, Lonza, Walkersville, MD). Cells were cultured for 7 to 12 days with media changes every 48 hours. These cells exhibited a typical “early outgrowth” phenotype by flow cytometry (i.e. CD45+, CD14+ and CD31+, with variable VEGFR2 expression). They were then subjected to electroporation (MaxCyte, Gaithersburg, MD) with pVAX1 plasmid containing the full cDNA for human eNOS. Cells were then replated and cultured overnight; subsequently adherent cells were lifted into suspension and prepared for injection. Cell viability in the final cell product was verified to exceed 80% by trypan blue exclusion and PVAX1/heNOS transfection was verified by qRT-PCR.

Delivery of eNOS gene-enhanced EPCs
Once satisfactory position of the distal port in the pulmonary artery was achieved, patients were acclimatized for at least 15 minutes before the first baseline readings of heart rate (HR), respiratory rate (RR), O2 saturation, blood pressure (BP), right atrial pressure (RAP), pulmonary artery pressure (PAP), mean PAP and pulmonary artery wedge pressure (PAWP) were recorded and cardiac output (CO) was measured in triplicate by thermodilution. After 10 minutes, full hemodynamic readings were repeated. Pulmonary vascular resistance (PVR), total pulmonary resistance (TPR) and systemic vascular resistance (SVR) were calculated.

A minimum waiting period of 2 weeks was required between enrolment of patients within the same dose panel, and a minimum period of 1 month between the last patient of a dose panel and the first patient in the next. Predefined stopping criteria are described below. Patient follow up visits were conducted at 1 week and 1, 3 and 6 months post cell delivery as detailed in table S1.

Stopping criteria: Dose escalation would be halted if: 1) a single patient had a SAE deemed “definitely” related to cell injection (death, life threatening adverse event); if 2 patients within a given dose panel had a SAE deemed “probably” related to cell injection; or if 3 patients within a given dose panel have a SAE deemed “possibly” related to cell injection. No further patients would be enrolled at this dose, and an additional 3 further patients would beenrolled at the immediate lower dose and to complete the trial.
Online Table I

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<th>Mo 12, 18, 24, 30, 36, 42, 48, 54, 60</th>
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Online Table II: Arterial blood gases during cell delivery

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<td>70 ± 15</td>
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<tr>
<td>GI related</td>
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<tr>
<td>Access site hematoma or pain</td>
<td>9 (10)</td>
</tr>
<tr>
<td>Respiratory</td>
<td>8 (9)</td>
</tr>
<tr>
<td>CBC abnormalities</td>
<td>6 (7)</td>
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<tr>
<td>Biochemistry</td>
<td>6 (7)</td>
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<tr>
<td>Anxiety or Fatigue related</td>
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<td>Inflammatory</td>
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<td>Palpitations</td>
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### Online Table IV: Pulmonary function tests

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</tr>
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<tbody>
<tr>
<td>FVC</td>
<td>85 ± 21</td>
<td>84 ± 19</td>
<td>82 ± 18</td>
</tr>
<tr>
<td>FEV1</td>
<td>76 ± 19</td>
<td>78 ± 17</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>DLCO</td>
<td>73 ± 24</td>
<td>81 ± 20</td>
<td>77 ± 14</td>
</tr>
</tbody>
</table>
Online Figure I: Absolute and relative changes in total pulmonary vascular resistance (TPR) in dose panels 1 (A), 2 (B) and 3 (C). Absolute values for each patient are shown the left-sided panels, whereas relative (%) change is presented on the right. Shaded bars represent each delivery day with pre- and post-cell injection data indicated by circle and squares, respectively. Open symbols denote patients that were receiving concomitant therapy with a phosphodiesterase type 5 inhibitor.
Online Figure II: Individual component scores of the Physical Component Summary (A. Physical Functioning (PF), B. Role-Physical, C. Body Plan and D. Global Health (GH)) and the Mental Component Summary (Vitality (VT)), at baseline and over a 6 follow up period. Statistical analyses were performed using general linear regression analyses.