Endothelial NO-Synthase Gene-Enhanced Progenitor Cell Therapy for Pulmonary Arterial Hypertension

The PHACeT Trial

John Granton,* David Langleben,* Michael B. Kutryk, Nancy Camack, Jacques Galipeau, David W. Courtman,* Duncan J. Stewart*

Rationale: Pulmonary arterial hypertension (PAH) remains a progressive and eventually lethal disease characterized by increased pulmonary vascular resistance because of 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 preclinical evaluation in experimental models of PAH.

Objective: The Pulmonary Hypertension and Angiogenic Cell Therapy (PHACeT) trial was a phase 1, dose-escalating clinical study of the tolerability of culture-derived endothelial progenitor cells, transiently transfected with endothelial nitric oxide synthase, in patients with PAH refractory to PAH-specific therapies.

Methods and Results: Seven to 50 million endothelial nitric oxide synthase–transfected endothelial progenitor cells, 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 intensive care unit setting. Seven patients (5 women) 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 toward improvement in total pulmonary resistance during the 3-day delivery period. However, there was 1 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, 6-minute walk distance was significantly increased at 1, 3, and 6 months.

Conclusion: Delivery of endothelial progenitor cells overexpressing endothelial nitric oxide synthase was tolerated hemodynamically in patients with PAH. Furthermore, there was evidence of short-term hemodynamic improvement, associated with long-term benefits in functional and quality of life assessments. However, future studies are needed to further establish the efficacy of this therapy.

Clinical Trial Registration: URL: http://www.clinicaltrials.gov. Unique identifier: NCT00469027.

Key Words: cell- and tissue-based therapy ■ endothelial progenitor cells ■ genetic therapy ■ hemodynamics ■ hypertension, pulmonary

Pulmonary arterial hypertension (PAH) is a devastating disease that is caused by a progressive increase in pulmonary vascular resistance, largely because of severe remodeling of distal lung arterioles. Recent evidence from experimental models has strongly implicated endothelial cell injury and apoptosis as a critical trigger for this disease. Loss of endothelial cells 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.

In This Issue, see p 585

Editorial, see p 596

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>CRP</th>
<th>C-reactive protein</th>
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<tr>
<td>eNOS</td>
<td>endothelial NO-synthase</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cell</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>NT-proBNP</td>
<td>N-terminal probrain natriuretic peptide</td>
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<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<tr>
<td>TPR</td>
<td>total pulmonary resistance</td>
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<td>WHO</td>
<td>World Health Organization</td>
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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, several 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 outgrowth EPCs (or circulating angiogenic cells) 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 nitric oxide synthase (eNOS) significantly enhanced the ability of EPC to reverse hemodynamic and remodeling abnormalities in the monocrotaline treatment model of established PAH. Even somatic cells, such as fibroblasts, were effective in improving pulmonary hemodynamics and microvascular perfusion in the monocrotaline treatment model when transfected with eNOS, consistent with the well-recognized role of NO in vascular repair and regeneration.

The Pulmonary Hypertension and Angiogenic Cell Therapy (PHACeT) trial 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 and sustained increases in exercise capacity.

Methods

The PHACeT trial was an open label, dose-escalating protocol. Patients were enrolled in 2 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 Data Monitoring and Safety Board (DSMB). All patients provided written informed consent. The inclusion criteria required that patients had World Health Organization (WHO) group 1 PAH; specifically, idiopathic, associated with systemic sclerosis, anorexigen exposure, or repaired atrial septal defect; WHO functional class III or IV symptoms despite treatment with conventional therapies, including intravenous epoprostenol at maximal tolerated doses; and 6-minute walk distance between 150 and 400 m. 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 Data Supplement.

Delivery of eNOS Gene-Enhanced EPCs

All patients were admitted to hospital electively for insertion of a multipor pulmonary arterial catheter via a central vein. The cell suspensions were injected by hand at a rate of ≤2 mL/min at a concentration of 2.5 million cells/mL. Full hemodynamics, including cardiac output and pulmonary artery wedge pressure, were recorded before and 30 minutes post each cell delivery as described in the Online Data 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 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 probrain 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), or the Quantikine HS ELISA for Human IL-6, #HS600B (R&D Systems, Inc, Minneapolis, MN).

Statistical Analysis

For this small, phase 1 study, we used descriptive statistics (means and SD) to assess safety and tolerability. To evaluate preliminary evidence of effectiveness, we used repeated measures ANOVA and
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 and GraphPad Prism (version 5 for Mac, GraphPad Software, La Jolla, CA, www.graphpad.com).

Results

Patient Characteristics

A total of 7 patients with idiopathic PAH were enrolled between November 2006 and March 2010. Five female and 2 male patients were included in the study, with a mean age of 52±20 years (Table 1). All patients had at least WHO class III symptoms, and their mean 6-minute walk distance was 361±110 m. All but the first patient were on dual oral PAH therapies (endothelin receptor antagonist and phosphodiesterase type 5 inhibitor) or intravenous epoprostenol with or without sildenafil. Table 2 summarizes the hemodynamic data obtained just before 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 before enrollment. Mean pulmonary arterial pressure at baseline was 55±13 mm Hg (median, 57 mm Hg), with a cardiac output of 4.91±1.87 L/m (median, 5.47 L/m) and a calculated total pulmonary resistance (TPR) of 1062±585 dynes/s·cm² (median, 800 dynes/sec·cm²; Table 2).

Short-Term Effects on Pulmonary Hemodynamics and Gas Exchange

Hemodynamic parameters were monitored before and after each cell infusion (Figure 2A and 2B). In the majority of patients, mean pulmonary arterial pressure and TPR declined during the 3-day cell delivery period, whereas cardiac output rose. The only exception was the patient with the highest cardiac output and the lowest pulmonary vascular resistance and TPR at baseline (patient 7, Table 2; Figure 2; double arrow). Overall, the decrease in TPR during the cell delivery period showed a statistical trend for all 7 patients (P=0.06). No cell dose–effect relationship was seen (Online Figure 1); 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 during 3-day delivery period. However, in absolute terms, the increase was modest, and thus the percent change was exaggerated because this patient had the lowest baseline TPR. Interestingly, the 3 patients receiving background sildenafil therapy, alone or in combination therapy, were among those with the greatest improvements in hemodynamics (Figure 2A–2C, open symbols), reaching significance for TPR (P<0.05; 2-way repeated measures ANOVA). Importantly, there were no significant changes in gas exchange as assessed by arterial O₂ partial pressure and saturation during the 3-day treatment period (Online Table II).

Adverse Events

Two serious adverse events occurred during the 1-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 presyncope and frequent admissions for right heart failure, in addition to other features consistent with poor prognosis (Tables 1 and 2). He was the second patient to receive a total of 23 million cells and showed no acute hemodynamic deterioration during or immediately after 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 computed tomographic 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 serious adverse event was for sepsis leading to hospitalization (patient 3), which occurred...

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>WHO FC</th>
<th>6MWD, m</th>
<th>DCLO, %</th>
<th>NT-proBNP, ng/L</th>
<th>PAH-Specific Medications</th>
<th>Other Medications</th>
</tr>
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<tbody>
<tr>
<td>01-001</td>
<td>75</td>
<td>F</td>
<td>III</td>
<td>354</td>
<td>65</td>
<td>1305</td>
<td>Bos, Spir, War</td>
<td></td>
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<tr>
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<td>F</td>
<td>III</td>
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<td>1361</td>
<td>Bos, Sild</td>
<td>War</td>
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<td>III</td>
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<td>250</td>
<td>Sild, Epo</td>
<td>Dig</td>
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<td>72</td>
<td>M</td>
<td>III</td>
<td>515</td>
<td>101</td>
<td>316</td>
<td>Epo</td>
<td>War</td>
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<tr>
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<td>68</td>
<td>M</td>
<td>III</td>
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<td>29</td>
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<td>418</td>
<td>88</td>
<td>80</td>
<td>Epo</td>
<td>War</td>
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</table>

6MWD indicates 6-minute walk distance; Bos, bosentan; DCLO, diffusing capacity or transfer factor of the lung for carbon monoxide; Dig, digoxin; Epo, epoprostenol; FC, functional class; Fur, furosemide; NT-proBNP, N-terminal probrain natriuretic peptide; PAH, pulmonary arterial hypertension; Sild, sildenafil; Sita, sitaxsentan; Spir, spironolactone; War, warfarin; and WHO, World Health Organization.

*Patient was not eligible for parental prostaglandin therapy because of the remote location of his residence.
9 months after cell delivery and was deemed unrelated to the cell product. After completion of the 1-year follow-up period, there were 2 more deaths, at 2.6 years (patient 2) and 4.6 years (patient 3) post cell delivery, as well as 1 patient who developed breast cancer 4 years post cell therapy. In addition, there were 6 hospitalizations occurring between 23 and 32 months

Table 2. Baseline Hemodynamics

<table>
<thead>
<tr>
<th>Patient</th>
<th>RAP, mm Hg</th>
<th>sPAP, mm Hg</th>
<th>dPAP, mm Hg</th>
<th>mPAP, mm Hg</th>
<th>CO, L/min</th>
<th>CI, L/m²</th>
<th>PaW, mm Hg</th>
<th>PVR, dynes/s</th>
<th>TPR, dynes/s</th>
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<td>46</td>
<td>22</td>
<td>31</td>
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<td>1.98</td>
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<td>116</td>
<td>45</td>
<td>75</td>
<td>3.3</td>
<td>1.54</td>
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<td>3.77</td>
<td>7</td>
<td>648</td>
<td>786</td>
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<tr>
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<td>57</td>
<td>5.47</td>
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<td>15</td>
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<td>5.83</td>
<td>3.19</td>
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<td>47</td>
<td>7.93</td>
<td>4.06</td>
<td>13</td>
<td>343</td>
<td>580</td>
</tr>
</tbody>
</table>

Mean±SD 8±5 86±23 38±8 55±13 4.9±1.9 2.64±1.06 12±6 713±536 1077±215

Bos indicates bosentan; CO, cardiac output; CI, cardiac index; Dig, digoxin; dPAP, diastolic pulmonary arterial pressure; Epo, epoprostenol; Fur, furosemide; mPAP, mean pulmonary arterial pressure; N/A, not available; PaW, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; RAP, right atrial pressure; Sild, sildenafil; Sita, sitaxsentan; sPAP, systolic pulmonary arterial pressure; Spir, spironolactone; TPR, total pulmonary resistance; and War, warfarin.

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

Figure 2. Acute hemodynamic changes in patients receiving cell therapy. Measurements of mean pulmonary arterial pressure (mPAP; A), cardiac output (CO; B), and total pulmonary resistance (TPR; C) were made using pulmonary arterial catheter pre and post cell infusion, on the 3 consecutive days of cell delivery immediately before cell injection (circles) and 30 minutes post cell injection (squares). Absolute values for each patient are shown in the left-sided panels, whereas relative (%) change is presented on the right. Open symbols denote patients who were receiving concomitant therapy with a phosphodiesterase type 5 inhibitor. Statistical analyses were performed using repeated measures ANOVA. No significant changes in mPAP or CO were noted; however, a trend toward improvement in TPR was observed during the 3-day course of cell infusion (P=0.06). –→ and => denotes patients 5 and 7, respectively.
post cell delivery involving 3 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 2 hospitalizations were for atrial fibrillation and a febrile condition, respectively, both occurring >2 years post treatment. Other adverse events that were reported during the study period are summarized in Online Table III. There was an average of 15±7 adverse events per patient (range, 5–24). The most frequently reported events were related to the musculoskeletal, gastrointestinal, and respiratory systems, as well as access site pain/hematoma; although these were for the most part minor.

Long-Term Functional, Quality of Life, and Hemodynamic Changes

Mean 6-minute walk distance improved significantly during the follow-up period (Figure 3A; *P* = 0.006), with a mean increase of 65 m at 1 month (*P* <0.001), persisting at 3 and 6 months (48 and 47 m, 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 m at 1 month (*P* = 0.001), persisting at 3 months (39 m; *P* = 0.012) and 6 months (38 m; *P* = 0.014). The statistically significant improvement in 6-minute walk distance persisted even after imputing a worsening of ≤20% for the patient who died (*P* = 0.032). Two patients demonstrated improvement in WHO functional class from class III to class II at 3 and 6 months of follow-up (Figure 3C) and this increased to 3 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 measure of the SF36 quality of life score (Figure 4; *P* <0.0001), whereas the mental component summary 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 6 patients who 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 6-month follow-up period, despite a tripling in levels on day 4 for 1 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 during 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 phosphodiesterase type 5 inhibitor as part of their background therapy, and also sustained benefits in exercise capacity, quality of life scores, and symptom class.
Although current pharmacological therapies for PAH provide benefit in terms of exercise tolerance and modest improvements in pulmonary hemodynamics,\textsuperscript{33–35} they do not seem to reverse the severe arteriolar remodeling which is characteristic of this disease\textsuperscript{36} and, with the exception of epoprostenol, their effect on survival has not been well established.\textsuperscript{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.\textsuperscript{39}

Cell therapy for other cardiovascular diseases has been studied in several 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 myocardial infarction,\textsuperscript{16,40} as well as long-term improvements in clinical outcomes, including event-free survival and mortality.\textsuperscript{17} Most cardiovascular cell therapy trials have used heterogeneous populations of bone marrow mononuclear cells,\textsuperscript{16,17,41} which are believed to harbor a rare population of stem or progenitor cells.\textsuperscript{42} Some studies have used progenitor cells selected on the basis of surface markers, such as CD34 or CD133,\textsuperscript{43,44} although there is no consensus about the definition of a true EPC population.\textsuperscript{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 mononuclear cells will attach and acquire a rod-like morphology by about 3 days of culture. These cells are termed early outgrowth EPCs\textsuperscript{46} or circulating angiogenic cells\textsuperscript{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.\textsuperscript{48} In that trial, EPCs were obtained from a simple peripheral blood draw yielding, on average, a total cell dose of $\approx$11 million EPCs after 7 days of culture (4–23 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 $\approx$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. All but 1 patient showed either no change or even an improvement in hemodynamics during the cell delivery period. No significant differences were seen in the 6 patients who completed follow-up.
growth factor and other angiogenic growth factors,52 as well as mediator of the angiogenic effects of vascular endothelial growth factor.53 Moreover, in a direct comparison with vascular cells, eNOS turned to baseline by the first week. However, the magnitude of these changes was modest compared with 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 whereas peak CRP levels were within the range as 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 products59 or xenobiotic reagents (fetal bovine serum),60 which could be expected to induce an immune response.

Although the acute cell delivery was hemodynamically well tolerated,1 patient died suddenly soon after discharge raising potential safety concerns, particularly in a patient population with severe baseline hemodynamic compromise. Although this patient exhibited several 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 think 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 among the highest of this population (Figure 6) and showed the greatest increases after cell delivery. Notably, this was the only patient who 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 (ie, recurrent admissions for right heart failure, presyncopal episodes, and excessive desaturation) and perform careful monitoring 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 <1 week. Although 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.21 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 1 month, but persisted for ≤6 months. This was associated with an improvement in quality of life scores during the course of follow-up and a decrease in 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 of 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 toward 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.

Acknowledgments
We are tremendously grateful for the assistance of Dr Alexander B. Zhai (Ottawa Hospital Research Institute [OHRI]), who was invaluable in helping draft the article. We would like to express our sincere gratitude to Joanna Sloninko (St. Michael’s Hospital [SMH]) and Samantha Hodgins (OHRI) who were instrumental in helping with data retrieval analysis. As well, we are indebted to Rima Hosn, study coordinator, and Mary McCarthy and Laura Greene, nurse coordinators at University Health Network (UHN), as well as Eileen Shalit, RN, Lyda Lesenko, Laura Greene, nurse coordinators at University Health Network (UHN), and Stephanie Fuoco, RN, nurse coordinators and Dr Andrew Leong-Poi and Abdul Al-Heysayen at SMH. Also, we are grateful for the expert statistical help and advice of Monica Taljaard and the Ottawa Hospital Research Institute Methods Centre. As well, we are indebted to Nicolette Eliopoulos and the talented staff in the cell manufacturing facilities at the JGH and UHN. We would also like to thank the members of the Data Monitoring and Safety Committee: Vallerie McLaughlin (Chair; University of Michigan), Sanjay Mehta (Western University), Robert Levy (University of British Columbia), and John Marshall (University of Toronto).

Sources of Funding
The study was sponsored by Northern Therapeutics Inc. D.J. Stewart 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.

Disclosures
D.J. Stewart is the president of Northern Therapeutics and has an equity interest in Northern Therapeutics. D.W. Courtman is the Chief Scientific Officer of Northern Therapeutics. The other authors report no conflicts.

References


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**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 Pulmonary Hypertension and Angiogenic Cell Therapy (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 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 pressure. There were significant and persistent benefits in the mean walking distance and quality of life scores for ≤6 months. This study suggests feasibility of administering genetically modified endothelial nitric oxide synthase to patients with severe PAH without immediate adverse hemodynamic effects. Future clinical trials are needed to support the use of this innovative approach in restoring functional lung blood vessels, as a potential transformative therapy, in patients with PAH.
Endothelial NO-Synthase Gene-Enhanced Progenitor Cell Therapy for Pulmonary Arterial Hypertension: The PHACeT Trial

John Granton, David Langleben, Michael B. Kutryk, Nancy Camack, Jacques Galipeau, David W. 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 $\geq 18$ years, $\leq 80$ 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 $\geq 150$ meters and $\leq 400$ 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 $\beta$-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 $>38.5^\circ C$, 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 (FEV$_1$/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
- h-eNOS 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 CD 31+, 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>Time point</th>
<th>Baseline phase</th>
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<th>Pre D/C</th>
<th>Wk 1 post D/C</th>
<th>Mo 1 post cell delivery</th>
<th>Mo 3 post cell delivery</th>
<th>Mo 6 post cell delivery</th>
<th>Mo 12, 18, 24, 30, 36, 42, 48, 54, 60</th>
<th>Every 12 months for added 10 years (total 15 yrs)</th>
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<td>Borg dyspnea index</td>
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<td>pVAX1-heNOS cell therapy delivery (note³)</td>
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<td>X*</td>
<td>X*</td>
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<td>Assessment of Adverse events &amp; SAEs</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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### Online Table II: Arterial blood gases during cell delivery

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<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<tr>
<td>PaO₂</td>
<td>68 ± 19</td>
<td>70 ± 15</td>
<td>74 ± 17</td>
<td>74 ± 18</td>
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<tr>
<td>O₂ Sat</td>
<td>92 ± 3</td>
<td>93 ± 6</td>
<td>94 ± 4</td>
<td>94 ± 4</td>
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<tr>
<td>PaCO₂</td>
<td>37 ± 4</td>
<td>36 ± 5</td>
<td>34 ± 4</td>
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<tr>
<td>pH</td>
<td>7.44 ± 0.03</td>
<td>7.43 ± 0.04</td>
<td>7.44 ± 0.04</td>
<td>7.45 ± 0.04</td>
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### Online Table III: Adverse Events

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<th>Category</th>
<th>Total</th>
<th>(%)</th>
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<td>Musculoskeletal related</td>
<td>11</td>
<td>(12)</td>
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<tr>
<td>GI related</td>
<td>10</td>
<td>(11)</td>
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<tr>
<td>Access site hematoma or pain</td>
<td>9</td>
<td>(10)</td>
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<tr>
<td>Respiratory</td>
<td>8</td>
<td>(9 )</td>
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<tr>
<td>CBC abnormalities</td>
<td>6</td>
<td>(7 )</td>
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<tr>
<td>Biochemistry</td>
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<td>(7 )</td>
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<tr>
<td>Anxiety or Fatigue related</td>
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<tr>
<td>Inflammatory</td>
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<td>(4 )</td>
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<td>Palpitations</td>
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<td>Dizziness</td>
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<td>Hypo or Hypertension</td>
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<td>(3 )</td>
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<tr>
<td>ENT related</td>
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<td>(3 )</td>
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<td>Eye related</td>
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<td>Skin related</td>
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<td>Neurologic related</td>
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<td>Headaches</td>
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<tr>
<td>Peripheral related</td>
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<td>Miscellaneous</td>
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### Online Table IV: Pulmonary function tests

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<th>6M</th>
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<td>FVC</td>
<td>85 ± 21</td>
<td>84 ± 19</td>
<td>82 ± 18</td>
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<td>FEV1</td>
<td>76 ± 19</td>
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<td>75 ± 16</td>
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<tr>
<td>DLCO</td>
<td>73 ± 24</td>
<td>81 ± 20</td>
<td>77 ± 14</td>
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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.