Studying the Effect of Cell Therapy by Endothelial Progenitor Cells With and Without Heme-oxygenase Inducers in Induced Pulmonary Hypertension Rat Model

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Abstract

Purpose: We investigated whether or not intravenous injection of bone marrow-derived endothelial progenitor cells (EPCs), alone or combined with Heme-oxygenase (HO) inducer can restore pulmonary hemodynamics and increase microvascular perfusion in the rat monocrotaline (MCT) model of pulmonary artery hypertension (PAH) so improve right ventricle performance.

Material and Methods: Rats were divided into control, rats with PAH, PAH rats receiving EPCs, PAH rats receiving HO-1 inducer (cobalt protoporphyrin) and PAH rats receiving EPCs plus HO-1 inducer. After 1 month, right ventricular function, was assessed. Gene expression of pro ANP, SDF-1, Bax/Bcl-2, MMP-9 were studied.

Results: Heart functions showed improvement in all the treated groups compared to PAH group. Gene expression demonstrated that proANP, MMP-9 and Bax were downregulated while Bcl-2 and SDF-1 were upregulated.

Conclusion: The present study proved that, administration of BM-EPCs alone, HO-1 alone or EPCs plus HO-1 produce therapeutic effect in restoring pulmonary hemodynamics and so improving right ventricle performance in PAH. This effect may be through antiapoptotic action of EPCs and HO-1.

Key Words: Pulmonary artery hypertension – EPCs and HO-1.

Introduction

PULMONARY endothelial dysfunction plays a prominent role in the pathogenesis of pulmonary arterial hypertension. Endothelial dysfunction leads to an imbalance in the production of vasoconstrictors e.g. endothelin-1 (ET-1) and Thromboxan versus vasodilators e.g. NO and prostacyclin, factors affecting smooth muscle cells [1]. This imbalance contributes to vasoconstriction and vascular remodelling with narrowing of pulmonary arterioles. In addition, endothelial cell (EC) injury and apoptosis have been implicated as critical triggers for the development of PAH [2].

Endothelial progenitor cells were shown to derive from bone marrow and to incorporate into foci of physiological or pathological neovascularization [3]. They have been reported to promote angiogenesis in vitro. So, they are seen as promising cells for the treatment of many vascular diseases, including PAH [4].

HO-1 is the inducible form of heme oxygenases that metabolize heme to carbon monoxide (CO), iron and biliverdin, which is converted to bilirubin by biliverdin reductase [8]. Rising evidence suggests that upregulation of HO-1 protects against vascular diseases, including atherosclerosis and intimal hyperplasia [6]. HO-1 is thought to protect against PAH via promoting reendothelialization, inducing antiinflammatory activities, inhibiting smooth muscle cell proliferation, regulating vascular tone, and by increasing cellular antioxidant activities [6]. HO-1 contributes to vascular repair also by increasing circulating EPCs derived from the bone marrow [7].

The aim of the work was to investigate whether or not intravenous injection of bone marrow-derived endothelial progenitor cells (EPCs), alone or combined with HO inducer can restore pulmonary hemodynamics and increase microvascular perfusion in the rat monocrotaline (MCT) model of PAH so improve right ventricle performance.

Material and Methods

This study was performed at the Unit of Biochemistry and Molecular Biology at the Medical
Biochemistry Department, Faculty of Medicine, Cairo University, Egypt during the period from March 2013 to May 2014.

**Preparation of the animal model:**

This study included fifty healthy, male adult white albino rats (200-250g body weight). Animals were inbred in the Experimental Animal Unit, Faculty of Medicine, Cairo University. Rats were bred and maintained in an air conditioned animal house with specific pathogen free conditions and were subjected to a 12:12-h daylight/darkness. Animals were fed a semi-purified diet that contained (gm/kg): 200 casein, 555 sucrose, 100 cellulose, 100 fat blends, 35 vitamin mix and 35 mineral mix. All animal experiments received approval from the institutional animal care committee.

**Animals were divided into 5 groups (Table 1):**

- PAH was induced in 40 rats by single s.c. injection of monocrotalline (MCT) (Sigma-Aldrich, Zwijndrecht, the Netherlands; 60mg/kg, after 14 days these rats were subdivided into 5 groups (10 rats each).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(10 rats each)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Healthy control.</td>
</tr>
<tr>
<td>Group 2</td>
<td>Positive control (PAH): Received 1ml PBS I.V.</td>
</tr>
<tr>
<td>Group 3</td>
<td>PAH+EPCs: Received 10^6 EPCs per rat given by Intravenous injection at the rat tail vein [8].</td>
</tr>
<tr>
<td>Group 4</td>
<td>PAH+HO: Received HO-1 inducer (cobalt protoporphyrin 0.5mg/100gm intraperitoneally, 3 times/week for 2 weeks) [9].</td>
</tr>
<tr>
<td>Group 5</td>
<td>EPCs +PAH+ HO.</td>
</tr>
</tbody>
</table>

- Age-matched control rats were injected with an equal volume of PBS intravenously (i.v).
- At the planned time; 1 month after therapy, venous blood was collected from the retro-orbital vein from rats of all groups to assess serum creatine phosphokinase enzyme. This was followed by sacrificing the animals (by CO² narcosis) and heart was taken out for assessment of:
  - Cardiac physiological parameters.
  - Pro ANP, SDF-1, Bax/Bcl-2, MMP-9 gene expression by real time PCR (in right ventricle RV).

**Preparation and Identification of BM derived EPCs from rats:**

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old white albino male rats with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] centrifugation. Cells were then cultured onto fibronectin coated plates suspended in medium supplemented with 20% fetal calf serum (FCS), 0.1% human vascular endothelial growth factor-1 (VEGF-1) and 0.1% insulin-like growth factor (IGF-1) at 37°C for 48 hours, after which the supernatant was removed [10]. Cells were incubated at 37°C in 5% humidified CO2 for 4-7 days as primary culture or upon formation of large colonies. When large colonies developed (80-90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) for 5min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50cm² culture flasks (Falcon). The resulting cultures were referred to as first passage cultures [11].

After seven days, cells were stained and labelled with 1, 1'-diocatadecyl- 3, 3', 3'-tetramethyldiocarbocyanine-labeled acetylated LDL (DiLDL) and FITC-labelled Ulex europaeus agglutinin I (UEA-1, Sigma Chemical Company); this double staining is specific for EPCs identification. Cells were counterstained with 4’, 6-diamidino-phenylindole (DAPI; 0.2µg/ml in 10mmol/l Tris-HCl, pH 7.0, 1mmol/l EDTA and 100mmol/l NaCl) for 10 minutes and cells visualized with a distinct blue cytoplasm under inverted fluorescent microscope; DAPI staining is used to ensure cells viability. Only double stained cells (DiLDL-FITC labelled UEA-1) with a distinctly blue cytoplasm (DAPI positive cells) were counted in a five random fields under fluorescent inverted microscope (Lieca, Germany) [10].

Flow Cytometry Analysis (FACS analysis) for CD34+ of cultured EPCs was done as an identification surface marker of EPCs.

**Assessment of cardiac physiological parameters:**

The cardiovascular system was assessed using a Langendorff apparatus. Rats were anesthetized with an intraperitoneal injection of sodium thiopental (10mg/100g body weight). Hearts were rapidly excised and immersed in ice-cold Krebs-Henseleit solution containing heparin (5000u) at ambient temperature (25°C), then mounted on the aortic cannula and subsequently perfused according to the Langendorff technique with Krebs Henseleit medium at a hydrostatic pressure of 55cm H²O and bubbled with a mixture of 95% O₂ and 5%
CO₂. The time between extraction of the hearts and their attachment to the Langendorff apparatus did not exceed 2min.

The temperature of the heart and of the perfusion medium was maintained at $37^\circ C$ by an external water bath. A latex balloon filled with saline was introduced into the left ventricle and was connected to a pressure transducer to monitor performance. We measured the intraventricular balloon volume needed to increase end diastolic pressure from 0 to 10-15mmHg; afterwards the balloon volume was kept constant.

After 30 minutes of stabilization, ischemia was induced by abolishing the coronary flow from (15ml/min) to 0ml/min for 30 minutes. The hearts were then reperfused for 60 minutes with a coronary flow of 15ml/min [12].

Mechanical performances of the left ventricle of the heart was determined by the systolic pressure, the heart rate and the peak rate of maximum left ventricular pressure rise $(dp/dt)$ which is a good index of contractility. Heart rate was also measured. These mechanical performance parameters were monitored during the experiment by a balloon inserted into the left ventricle and connected to a polygraph apparatus (San-ei Instruments, Ltd.).

Serum creatine phosphokinase (CPK) estimation:

Venous blood was collected from the retro-orbital vein from rats of all groups, left to clot for 30 minutes, and centrifuged at 10,000rpm for 20 minutes. Serum CPK was measured by using EnzyChromTM Creatine Kinase Assay Kit (ECPK-100).

Quantitative real time (QRT) PCR for pro ANP, SDF-1, MMP-9, Bax and Bcl-2 in heart tissue:

Total RNA was extracted from cardiac tissues homogenate by using SV Total RNA Isolation System supplied by Promega (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Extracted RNA was quantified by spectrophotometer at 260nm.

The extracted RNA was reversed transcribed into cDNA using high capacity Reverse Transcription System kit (#K1621, Fermentas, USA). cDNA was generated from 1 µg of total RNA extracted according to manufacturer instructions.

The relative abundance of mRNA species was assessed using the SYBR Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank (Table 2). All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in duplicate in a 25 µl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900nM of each primer and 2-3 µg of DNA. Amplification conditions were 2min at 50°C, 10min at 95°C and 40 cycles of denaturation for 15s and annealing/extension at 60°C for 10min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of pro ANP, SDF-1, MMP-9, Bax and bcl2 mRNA was calculated using the comparative Ct method. All values were normalized to the beta actin genes and reported as fold change.

Table (2): Oligonucleotide primers sequence used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>ProANP</td>
<td>Forward primer: 5'-GAAAAGCACAATCTGAGGCTCTG-3'. Reverse primer: 5'-CCTAACCAGCAAGCAGCT-3'.</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Forward primer: 5'-CTTTGTCAGGGCATAATCTCA-3'. Reverse primer: 5'-TGTGATTTGACCGAAATTA-3'.</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward primer: 5'-AAG AGG CCT GGT TAC CCT GT-3'. Reverse primer: 5'-AAG TAG CAC CTG GGA GGG AT-3'.</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward primer: 5'-GTGACCTCTTCTTACTTTG-3'. Reverse primer: 5'-AGCCACCTCTGGTCTTG-3'.</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Forward primer: 5'-CTGGACACGGGTATGA-3'. Reverse primer: 5'-CGGGGTGAGGAGAAGAT-3'.</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward primer: 5'-TGCTGTTGGCTGATGATGTCG-3'. Reverse primer: 5'-TGGAGAGCaATGCCAGCC-3'.</td>
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</tbody>
</table>

Statistical analysis:

Data were expressed as mean±SD. Significant differences were determined by using ANOVA and post-hoc tests for multiple comparisons using SPSS 16 computer Software. Results were considered significant at $p<0.05$. 
Results

Hemodynamic measurements and serum CPK level:
Hemodynamic measurements (Mean±SD) of Heart rate, right ventricular systolic pressure (RVSP) and contractility was measured in all groups. Heart functions showed improvement in all the treated groups compared to PAH group [(Table 3) and Figs. (1-3)]. The level of CPK-MB in the serum of the MCT induced pulmonary artery hypertensive (PAH) (diseased) group shows a significant increase compared to the control healthy group which confirmed the cardiac affection in PAH (Table 4).

Quantitative real time (QRT) PCR:
Quantitative real time (QRT) PCR for pro ANP, SDF-1, MMP-9, Bax and Bcl-2 in heart tissue was done. Gene expression demonstrated that proANP, MMP-9 and Bax were downregulated while Bcl-2 and SDF-1 were upregulated in all treated groups Figs. (4-7).

Table (3): Hemodynamic measurements (Mean±SD) of Heart rate, right ventricular systolic pressure (RVSP) and contractility.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PAH</th>
<th>EPCs only</th>
<th>HO only</th>
<th>EPCs+ HO</th>
</tr>
</thead>
<tbody>
<tr>
<td>heart rate (beat/min)</td>
<td>162.61±2.99</td>
<td>108.1±3.52</td>
<td>140.67±1.28</td>
<td>132.8±3.13</td>
<td>139.37±0.52</td>
</tr>
<tr>
<td>systolic pressure (mmHg)</td>
<td>115.33±2.36</td>
<td>155.1±2.52</td>
<td>122.09±1.23</td>
<td>133.4±2.79</td>
<td>129.08±2.17</td>
</tr>
<tr>
<td>contractility index (mmHg/sec)</td>
<td>148.04±5.82</td>
<td>87.39±4.86</td>
<td>129.39±1.85</td>
<td>117.41±1.04</td>
<td>121.87±2.25</td>
</tr>
</tbody>
</table>

Values are represented as mean±SD.
* : Statistically significant compared to corresponding value in –ve control group (p<0.05).
# : Statistically significant compared to corresponding value in +ve control group ( p<0.05).
$ : Statistically significant compared to corresponding value in EPCs only group (IIB) (p<0.05).
&: Statistically significant compared to corresponding value in HO only group (p<0.05).
Table (4): CPK (Mean±SD) level (u/l) in rat serum of studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Control n=10 (-ve control)</td>
<td>119.54±2.74</td>
</tr>
<tr>
<td>2- PAH n=10 (+ve control)</td>
<td>246.63±26.99*</td>
</tr>
<tr>
<td>3- PAH+EPCs n=10.</td>
<td>149.49±16.63 *#</td>
</tr>
<tr>
<td>4- PAH+HO n=10.</td>
<td>167.83±18.01 *#</td>
</tr>
<tr>
<td>5- PAH+EPCs+HO n=10.</td>
<td>155.93±15.98 *#</td>
</tr>
</tbody>
</table>

EPCs isolation, propagation and identification:

A- EPCs in culture:

EPCs were isolated from rat bone marrow, cultured and propagated for 7 days on fibronectin coated wells using media supplemented with specific growth factors as; VEGF-1 and IGF-1 Fig. (8).

Fig. (5): Comparison between the MMP-9 relative gene expressions in rat heart tissue of studied groups.

Fig. (6): Comparison between the SDF-1 relative gene expression in rat heart tissue of studied groups.

Fig. (7): Comparison between the Bax and Bcl-2 relative gene expression in rat heart tissue of studied groups.

Fig. (8): EPCs in culture. (A): 0 day. (B): 4 th day.
**EPCs CFU characterization in culture:**

EPCs were characterized in culture by formation of CFU.

**B- Analysis of EPCs based on cell surface marker expression:**

EPCs were characterized by their surface positive expression of CD34 Fig. (9).

**C- EPCs characterization by specific fluorescent stains:**

EPCs were characterized by their specific double fluorescent staining with DiLDL-UAE-1 stains. EPCs stained with DAPI to ensure their viability and survival in vitro.

[Graph: CD34+ Counts]

**Discussion**

The pulmonary vascular remodeling in PAH involves proliferation of endothelial and smooth muscle cells, fibrosis, thrombosis and inflammation. This results in the formation of PAH-specific neointimal and more complex plexiform lesions leading to the obstruction of the pulmonary vessel lumen [13]. It has been reported that EPCs are increased in lung diseases with high pulmonary arterial pressure (PAP) suggesting an involvement in pulmonary vascular remodelling at end-stage pulmonary disease [14,15]. EPCs are seen as promising cells for the treatment of many vascular diseases, including PAH [16]. Alterations occurring with the HO/CO system in pulmonary arteries in hypertension were examined, leading to the conclusions that an impaired HO/CO-sGC/cGMP system in the pulmonary arteries of young and prehypertensive subjects might be indicative of the pathogenesis and development of hypertension [17].

In the present study, we aimed to investigate the effect of intravenous injection of BMEPCs, alone or combined with HO inducer in restoring pulmonary hemodynamics and increasing microvascular perfusion in the rat monocrotaline (MCT) model of PAH so improving right ventricle performance.

There was a significant increase in the level of CPK-MB in the serum of the MCT induced pulmonary artery hypertensive (PAH) (diseased) group when compared to the control healthy group which confirmed the cardiac affection in PAH.

Endogenous atrial naturureteic peptide (ANP) plays a physiological role in modulating pulmonary arterial pressure, cardiac hypertrophy, and pulmonary vascular remodeling under normoxic and hypoxic conditions [18]. It is produced in cardiac atrial tissues and causes natriuresis, diuresis, vasodilation and inhibition of aldosterone secretion. In the present study, there was a significant increase in the proANP gene expression in the diseased (PAH) group compared to the control healthy group. This agrees with Wiedemann and co-workers [19] who found that ANP system is highly activated in patients with severe persistent PH and non persistent PH and its level is significantly correlated with parameters of RV function and pre- and afterload. In the present study, when the BM-EPCs were I.V injected in the MCT induced model, there was a significant decrease in proANP level when compared to the PAH group. When HO-1 inducer (cobalt) was intraperitonealy injected in the MCT model, there was a significant decrease in proANP level when compared to the PAH group. There was significant decrease in the proANP levels in the group received EPCs combined with HO-1 inducer in comparison to the PAH group.

Matrixmetalloproteinase-9 (MMP-9) (gelatinase B) expression by cardiac fibroblast is very low under basal conditions, but is markedly increased in response to proinflammatory cytokines and oxidative stress [20]. In the present work, there was significant increase in MMP-9 expression in PAH group compared to the control group. This agrees with Safdar et al., [21] who found that circulating level of MMP-9 was higher in the PAH group as compared with age-and sex-matched healthy controls. In the present study, when the BM-EPCs were introduced whether alone or combined with HO-1 inducer showed significant decrease in MMP-9 gene expression compared to the PAH group.

SDF-1 has a significant role in regulating homing of stem cells and in maintaining the stem cell niche under steady state homeostasis [22]. In our study, there was significant increase in SDF-1 gene
expression in all treated groups compared with PAH group with no significant difference between the groups received EPCs alone or combined with HO inducer.

Apoptosis is an important mechanism maintaining homeostasis during development and for response to external stimuli in multicellular organisms [23]. The mitochondrial pathway of apoptosis is mediated by the release of a number of factors from mitochondria. The release of cytochrome c is the central gate in turning on or off apoptosis and is regulated by the interaction of proapoptotic proteins, including Bax which is taken as indices of apoptotic cell death and Bcl-2 which is taken as an index of anti-apoptotic factor [23]. In the present study, bax gene expression was significantly increased in the PAH group compared to the control group.

The proto-oncogene Bcl-2 can inhibit apoptosis in biological systems [24,25]. In the present work, there was a significant decrease in the Bcl-2 gene expression in the PAH group when compared to the control group. In the present study, all treated groups showed significant increase in Bcl-2 gene expression compared to PAH group.

Haemodynamic measurements were done, the PAH group showed significantly increased right ventricular systolic pressure (RVSP) while the heart rate (HR) and contractility index (CI) were decreased compared to the control group. All the treated groups showed significant decrease in RVSP and increase in both HR and CI compared to PAH group.

In conclusion, the present study proved that, administration of BM-EPCs alone, HO-1 alone or EPCs plus HO-1 produce therapeutic effect in restoring pulmonary hemodynamics and so improving right ventricle performance in PAH. This effect may be through antiapoptotic action of EPCs and HO-1.

References


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